Expression and Characterization of Ligand Binding by the Ectodomain of Toll-like Receptor 9

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By

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ABSTRACT

Toll-like receptor 9 (TLR9) activates the innate immune system in response to microbial DNA or mimicking oligodeoxynucleotides. While the discrimination of host and microbial DNA is presumed to reflect TLR9-mediated recognition of CpG motifs, little information is available to verify this hypothesis. Cell stimulation experiments demonstrate preferential activation of TLR9 by CpG-containing nucleic acids, however direct binding investigations have reached contradictory conclusions with respect to the ability of TLR9 to bind nucleic acids in a sequence-specific fashion. Here we report expression of the soluble, ectodomain of human TLR9 with characterization of its ligand-binding properties. TLR9 has a high degree of ligand specificity in being able to discriminate not only CpG dinucleotides, but also higher order six nucleotide motifs that mediate species-specific activation. However, TLR9 ligand binding is also functionally influenced by nucleic acids in a sequence-independent manner both *in vitro* and in cell proliferation experiments. A model is proposed in which TLR9 activation is mediated specifically by CpG-containing ligands while sensitivity is mediated specifically by the absolute concentration of nucleic acids in a sequence-independent manner.

The bovine hsp70A promoter was used to direct the heat-regulated synthesis of the ectodomain of human TLR9 in transfected cultured bovine cells. The protein was efficiently secreted from transfected cells in a temperature-dependent manner and the recombinant receptor produced was found to be relatively pure. A stably transfected cell line with regulated expression of the protein was obtained and repeated thermal cycling of the cultures enabled high-yield production of the receptor in an active ligand-binding form. Using this recombinant receptor to study the ligand binding properties of

TLR9, a model of positive cooperativity is proposed in which the sensitivity of TLR9 ligand binding is modulated by the absolute concentration of nucleic acids in a sequence-independent fashion, while activation of TLR9 is highly dependent on DNA sequence. That is to say that TLR9 is 'primed' for activation by interaction with non-activating sequences but activation itself occurs in a sequence-specific fashion.

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ABBREVIATIONS USED

AP-1	activating protein 1
APC	antigen presenting cell
BCIP/NBT	5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium
CARD	caspase activating and recruitment domain
CpG	cytosine-phosphate-guanosine
DAPI	4'-6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DOTAP	*N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propan methylsulfate
DTT	dithiothreitol
(ds, ss)DNA	(double-stranded, single-stranded) deoxyribonucleic acid
ER	endoplasmic reticulum
EST	expressed sequence tag
FBS	fetal bovine serum
GpC	guanosine-phosphate-cytosine
HEK	human embryonic kidney cells
hsp60	heat-shock protein 60
hsp70A	heat-shock protein 70A
HSV	herpes simplex virus
IκB	inhibitor of kappaB
IKK	IκB kinase
IL	interleukin
IL-1R	interleukin-1 receptor

IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LTA	lipoteichoic acid
LTR	long terminal repeat
MDBK	Madin-Darby bovine kidney cells
MDP	muramyl dipeptide
meso-DAP	meso-diaminopimelate
MHCI/II	major histocompatibility complex class I/II
MyD88	myeloid differentiation primary response gene 88
NACHT	neuronal apoptosis inhibitory protein, MHC class II transcription activator, incompatibility locus protein from *Podospora anserina*, and telomerase-associated protein
NAD	NACHT-associated domain
NALP	neuronal apoptosis inhibitory protein, MHC class II transcription activator, incompatibility locus protein from *Podospora anserina*, and telomerase-associated protein (NACHT)- leucine-rich repeat (LRR)-pyrin domain (PYD)-containing proteins
NF-κB	nuclear factor kappaB
NLR	NOD-like receptor
NOD	nucleotide oligomerization domain
ODN	oligodeoxynucleotide

PAMP	pathogen-associated molecular pattern
pCMV	pFLAG-CMV2 expression vector
PD	phosphodiester
pDC	plasmacytoid dendritic cell
PNGase F	peptide *N*-glycosidase F
poly I:C	polinosinic-polycytidylic
PRR	pattern recognition receptor
PTO	phosphorothioate
PYD	pyrin domain
(ds, ss)RNA	(double-stranded, single-stranded) ribonucleic acid
RNase	RNA endonuclease
SDS	sodium dodecyl sulfate
SPR	surface plasmon resonance
TBS(T)	Tris-buffered saline (with Tween-20)
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
$TLR^{-/-}$	TLR knockout
TNF	tumor necrosis factor

1.0 LITERATURE REVIEW

1.1 Mammalian Immunity

The mammalian immune system protects the host from infection through the identification and neutralization of pathogens. The difficulty inherent in this task is that pathogens present themselves in a variety of forms, ranging from parasites to bacteria, and these threats must be detected with specificity amongst normal tissues and cells. In addition, the immune system has also served as an evolutionary pressure to drive pathogens to evolve active strategies to avoid detection by the immune system (Basset, Holton et al. 2003; Ulevitch 2004). To meet these challenges, the mammalian immune system has evolved multiple mechanisms and overlapping systems to ensure efficient recognition of a diverse spectrum of potential microbial invaders. These immune systems consist of two distinct, but interconnected and complimentary branches – adaptive and innate immunity.

The innate and adaptive immune systems protect the host from the threat of infection with layered defenses of increasing specificity. If a pathogen breaches the body's physical barriers, the innate immune system provides an immediate, non-specific response. Innate immunity therefore refers to non-specific defense mechanisms that are activated immediately upon recognition of biomolecules associated with microbes (Hoffmann, Kafatos et al. 1999). If a pathogen escapes the defenses of the innate immune system, the adaptive immune system is then activated to initiate

responses which are specific to that pathogen. This system adapts, or changes, over time in order to recognize particular pathogens more effectively. Adaptive immunity creates immunological memory, which allows more effective and efficient protection against pathogens previously encountered by the host (Hoebe, Janssen et al. 2004; Tosi 2005). Adaptive immunity is defined by antigen-specific immune responses, specifically those generating immunological memory for rapid clearance of pathogens already encountered by the system. These two systems do not function as distinct entities, as the adaptive immune system is activated by the innate immune system (Medzhitov and Janeway Jr. 1997).

1.1.1 Innate and Adaptive Immunity

Innate immunity is the first line of host defense response which acts immediately following infection to prevent further invasion by the pathogen (Hoffmann, Kafatos et al. 1999). Recognition of foreign molecules/antigen by the innate immune system, and distinguishing self molecules from non-self, relies on pattern recognition receptors (PRRs). These receptors recognize conserved microbial molecular patterns which are highly conserved across a wide range of bacterial and viral species and are largely absent from the host anthology of biomolecules (Medzhitov and Janeway Jr. 1997). The innate immune system is made up of cells and signaling pathways which defend the host from infection by foreign organisms. The cells, and associated response mechanisms, recognize and respond to pathogens non-specifically and do not confer long-term protective immunity to the host. The innate immune system responses involve various effector mechanisms including, but not limited to, phagocytosis by macrophages, the

production of antimicrobial compounds and the production of cytokines and chemokines which mediate the recruitment of inflammatory cells (Hoffmann, Kafatos et al. 1999; Janeway Jr. and Medzhitov 2002).

The innate immune system is an evolutionarily older defense mechanism than the adaptive immune system, and it is present in insects, plants, fungi, and other primitive organisms (Medzhitov and Janeway Jr. 2000). Whereas vertebrates also rely on the adaptive immune responses, invertebrates rely solely on the mechanisms of the innate immune system to combat infection – a testament to the strength of the innate immune system. The major functions of the vertebrate innate immune system include (Murray, Rosenthal et al. 2002):

- activation of mechanisms, such as the complement cascade, to identify bacteria and to activate cells which promote clearance of dead cells or antibody complexes
- recruitment of immune cells to the site(s) of infection
- inflammation via production of cytokines and chemokines
- identification and removal of foreign substances by specialized white blood cells (lymphocytes), and
- activation of the adaptive immune system through antigen presentation.

In contrast, adaptive immunity is activated in vertebrates when a pathogen successfully evades the innate immune system. While the details of activation of adaptive immunity are outside the scope of this thesis, the major functions of the adaptive immune system include (Janeway 2001):

- discrimination of self antigens from non-self during antigen presentation by the innate immune system
- generation of specific responses to eliminate specific pathogens or pathogen-infected cells, and
- development of immunological memory, whereby each pathogen is recognized by, or associated with, a signature antigen; memory cells can be called upon to readily eliminate a pathogen should subsequent infection occur.

1.1.2 How Adaptive Immunity Recognizes Non-Self

As the focus of this thesis is on how a specific component of the innate immune system, Toll-like Receptor 9 (TLR9), recognizes pathogens, only a brief description of the recognition mechanisms employed by the adaptive immune system will be presented as a point of comparison.

The adaptive immune system is composed of highly specialized cells and processes that eliminate pathogenic challenges (Chaplin 2003). The adaptive immune response provides the immune system with the ability to recognize and remember specific pathogens, to generate immunity, and to mount stronger attacks each time the pathogen is encountered (Hoebe, Janssen et al. 2004). This serves to prepare the body for future challenges. This ability relies on the diversity of antigen receptors, designed to specifically recognize a virtually unlimited panel of antigens. This is achieved by localized somatic mutations and recombination/rearrangement of gene segments within the B cell antigen receptors (Diaz and Flcjnik 1998). These gene rearrangements lead to irreversible changes in the DNA of each cell and, as such, all progeny of the original cell

will inherit genes encoding the same receptor specificity, including B and T memory cells, which are the key to long-lasting specific immunity (Janeway 2001; Janeway Jr. and Medzhitov 2002). This diversity allows a small number of genes to generate a large number of different antigen receptors that can be expressed uniquely on individual cells.

Adaptive immunity also relies on the capacity of immune cells to distinguish between the body's own cells and invading cells, as host cells express self antigens which are different from the non-self antigens found on the surface of bacterial cells or on virally-infected host cells (Medzhitov and Janeway Jr. 2000). The adaptive response is triggered by the recognition of these non-self antigens. The adaptive immune system of lymphocytes, which express a single receptor on each cell, is selected positively by self-antigens, and negatively by self-antigens which could potentially cause autoimmune disease (Medzhitov and Janeway Jr. 2000; Monroe, Bannish et al. 2003).

All nucleated cells expressing major histocompatibility (MHC) class I or II molecules have the capacity to activate the adaptive immune response through presentation of antigen (Janeway 2001). Different cell subsets, including dendritic cells (DCs), B cells and macrophages, express immunostimulatory receptors which allow enhanced activation of T cells. These are termed professional antigen presenting cells (APCs). Several subgroups of T cells can be activated by professional APCs, and each type of T cell is equipped to deal specifically with unique pathogens (Taams, van Eden et al. 1999). The type of response generated depends on the type of T cell activated, and this depends largely on the context in which the APC encounters the antigen.

1.1.3 How Innate Immunity Recognizes Non-Self

1.1.3.1 Pattern Recognition Receptors

In contrast to adaptive immunity, which has the potential to generate a virtually limitless number of protein recognition molecules specific to particular antigens, the innate immune system utilizes only a limited number of receptors for recognition of an equally limited number of targets. These receptors recognize the core building blocks from which their targets are constructed; nucleic acids (DNA and RNA) and protein components such as lipopolysaccharides, *etc*.

Recognition of non-self antigens by the innate immune system relies on germline-encoded pathogen recognition receptors, PRRs, which recognize conserved microbial molecular patterns (pathogen-associated molecular patterns, PAMPs) (Gordon 2002). PRRs are proteins expressed by cells of the immune system to identify molecules associated with microbial pathogens – PAMPs. These receptors are categorized according to their ligand specificities, function and location within the cell.

Two of the primary classes of PRRs can be described by their cellular location; the TLR family, which are membrane-associated, and the nucleotide-oligomerization domain (NOD)-like receptors (NLRs) which occupy cytoplasmic locations.

1.1.3.2 Nucleotide-oligomerization domain-containing receptors and NACHT-LRR-PYD containing proteins

The NLRs are cytoplasmic proteins that appear to function in the regulation of inflammatory responses and apoptotic pathways (Inohara, Chamaillard et al. 2005; Strober, Murray et al. 2006). The NOD-like nomenclature arises because all of the

members of this family contain nucleotide-oligomerization domains (NODs). Approximately twenty of these proteins have been identified in the mammalian proteome and include two major subfamilies called NODs and NALPs [neuronal apoptosis inhibitory protein, MHC class II transcription activator, incompatibility locus protein from *Podospora anserina*, and telomerase-associated protein (NACHT)-leucine-rich repeat (LRR)- pyrin domain (PYD)-containing proteins]. Current knowledge suggests that these families of proteins recognize microbial molecules or stress responses and form receptor oligomers to activate inflammatory responses. These responses include activation of inflammatory cytokines and/or activation of the NF-κB signaling pathway to induce production of other varieties of inflammatory molecules to initiate the inflammatory response (Chamaillard, Girardin et al. 2003; Fritz, Ferrero et al. 2006). These families of proteins have conserved structural and functional elements.

As shown in **Figure 1.1**, the NOD receptors are characterized by a C-terminal LRR domain, a central NACHT domain, followed by a NACHT-associated domain (NAD) and an N-terminal protein-protein interaction domain which is either a caspase activating and recruitment domain (CARD) or pyrin domain (PYD) (Inohara, Chamaillard et al. 2005; Fritz, Ferrero et al. 2006; Strober, Murray et al. 2006). Within the NLRs, the LRR represents the ligand-binding domain; similarly TLRs also use LRR domains to mediate ligand binding. The protein-protein interaction domains, either CARD or PYD, serve to mediate interactions with CARD- or PYD-containing intracellular adapter molecules, respectively, that initiate signal transduction events. Within the TLRs a similar requirement is fulfilled with the TIR domains.

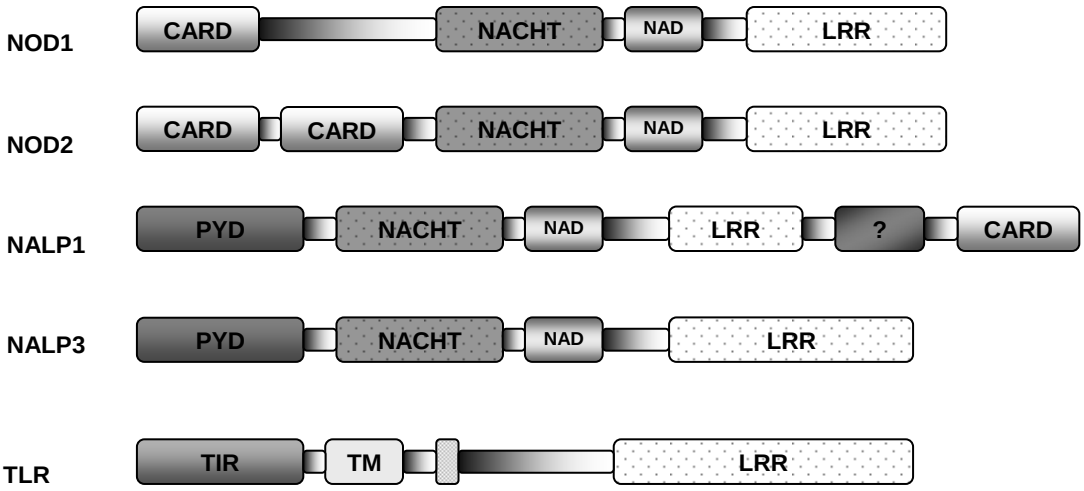

Figure 1.1. Domain comparison of pattern recognition receptors. NLRs (NOD, NALP) contain a protein-protein interaction domain, CARD or PYD, homologous to the TIR domain of TLRs. All PRRs contain LRR motifs required for ligand binding. CARD: caspase activating and recruitment domain, NACHT: neuronal apoptosis inhibitory protein, MHC class II transcription activator, incompatibility locus protein from *Podospora anserina*, and telomerase-associated protein, NAD: NACHT-associated domain, LRR: leucine-rich repeat, PYD: pyrin domain, TIR: Toll/interleukin-1 receptor, TM: transmembrane domain.

NOD1 contains CARD, LRR and NACHT domains. The adaptor protein Rip2, which is homologous to the TLR adaptor protein IRAK, also contains a CARD domain and leads to activation of NF-κB via the IKK signalsome (Chamaillard, Girardin et al. 2003). NOD2 is structurally very similar to NOD1, but contains two CARD domains, rather than one.

Currently, there are many NODs for which ligands have yet to be identified. There are few NODs to which the ligands have been assigned; NOD1 receptors recognize a molecule called meso-diaminopimelate (meso-DAP), a unique muropeptide peptidoglycan component found only in Gram negative bacteria. NOD2 receptors recognize intracellular muramyl dipeptide (MDP), which is the minimal bioactive peptidoglycan motif common to all bacteria (Franchi, McDonald et al. 2006).

Like NODs, NALPs contain C-terminal LRRs, which appear to act as regulatory domains involved in the recognition of invading pathogens. Also like NODs, these proteins contain a nucleotide binding site (the NACHT domain). Interaction with other proteins is mediated via N-terminal pyrin domain (PYD), rather than CARD. There are currently fourteen known members of the NALP subfamily. Activating ligands for NALPs include muramyl didpeptide, ATP, double-stranded RNA, and bacterial DNA (Strober, Murray et al. 2006) (**Table 1.1**).

In contrast to the recognition of cytosolic PAMPs by the NODs and NALPs, recognition of extracellular or endosomal PAMPs is mediated by the TLR family of receptors (Beutler, Jiang et al. 2006). These receptors are known to initiate a series of signaling pathways that culminate in the synthesis and secretion of cytokines and activation of other host defense programs central to the development of an immune

Table 1.1. Known ligands for known pattern recognition receptors. Ligands are known for the majority of the TLRs, while the ligands for most of the NLRs remain unknown. NOD1/2 and NALP1/3 are representative of this family of receptors.

Receptor	Known ligand(s)	Cellular Location
NOD1	Meso-diaminopimelate	Cytosol
NOD2	Intracellular muramyl dipeptide	Cytosol
NALP1	Muramyl dipeptide	Cytosol
NALP3	Muramyl dipeptide	Cytosol
TLR1	Triacyl lipoproteins	Plasma membrane
TLR2	Lipoproteins, Gram-positive peptidoglycan, lipoteichoic acids	Plasma membrane
TLR3	Double-stranded RNA, poly I:C	Endosome
TLR4	Lipopolysaccharide	Plasma membrane
TLR5	Bacterial flagellin	Plasma membrane
TLR6	Diacyl lipoproteins	Plasma membrane
TLR7	Single-stranded RNA, small synthetic compounds	Endosome
TLR8	Single-stranded RNA, small synthetic compounds	Endosome
TLR9	Unmethylated CpG DNA	Endosome
TLR11	Profilin	?

response (Doyle and O'Neill 2006). The TLRs, specifically TLR9, will be the focus of this thesis and will be discussed in greater detail.

1.2 Toll-like Receptors

TLRs are a family of type I transmembrane PRRs involved in recognition of a wide range of PAMPs, from bacterial DNA and cell wall components to viral RNA (Krutzik, Sieling et al. 2001; Janeway Jr. and Medzhitov 2002; Kaisho and Akira 2002; Underhill and Ozinsky 2002). The TLR family is highly conserved in vertebrates, with the identification of thirteen members in mammals and ten human homologues as of 2004 (Medzhitov, Preston-Hurlburt et al. 1997; Chaudhary, Ferguson et al. 1998; Rock, Hardiman et al. 1998; Takeuchi, Kawai et al. 1999; Chuang and Ulevitch 2000; Du, Poltorack et al. 2000; Chuang and Ulevitch 2001; Dunne and O'Neill 2003; Zhang, Zhang et al. 2004) (**Table 1.2**, **Table 1.3**). These receptors are composed of an extracellular domain, or ectodomain, which is characterized by repeating units of a leucine-rich motif, and an intracellular signaling domain that shares homology with the mammalian IL-1 receptor (IL-1R), known as the Toll/IL-1R (TIR) domain (**Figure 1.2**). Overall evidence would suggest that PAMPs trigger signaling responses through interaction with the ectodomain of these receptors leading to activation of NF-κB through conserved TLR signaling pathways. However, the mechanisms by which these ligands are recognized and how this recognition ultimately leads to TLR signaling are currently not well-understood.

Table 1.2. Number of amino acids, leucine-rich repeats and molecular weights of human Toll-like receptors 1-10. GenBank ascension numbers of the receptors are also included.

TLR	Amino acids	LRRs	MW (kDa)	Ascension #
1	786	18	84	U88540
2	784	19	84	U88878
3	904	24	97	U88879
4	839	22	90	H48602
5	858	23	91	U88881
6	796	20	91	AB020807
7	1049	27	121	AF240467, AF245702
8	1041	26	120	AF245703, AF246971
9	1032	27	116	AF245704, AF259262, AF259263
10	811	?	95	AF296673

Table 1.3. Sequence comparison of human Toll-like receptors 1-10. Values result from comparison of the entire sequences of all 10 TLRs and values are percent amino acid sequence identity. GenBank ascension numbers are as follows: TLR1 [U88540], TLR2 [U88878], TLR3 [U88879], TLR4 [H48602], TLR5 [U88881], TLR6 [AB020807], TLR7 [AF240467], TLR8 [AF245703], TLR9 [AF245704], TLR10 [AF296673].

TLR	1	2	3	4	5	6	7	8	9	10
1	**100**	29	23	25	22	68	21	23	22	48
2		**100**	22	26	23	31	24	24	25	29
3			**100**	24	26	23	26	26	24	24
4				**100**	24	25	23	24	24	23
5					**100**	23	24	25	24	22
6						**100**	22	22	22	46
7							**100**	43	36	22
8								**100**	35	21
9									**100**	21
10										**100**

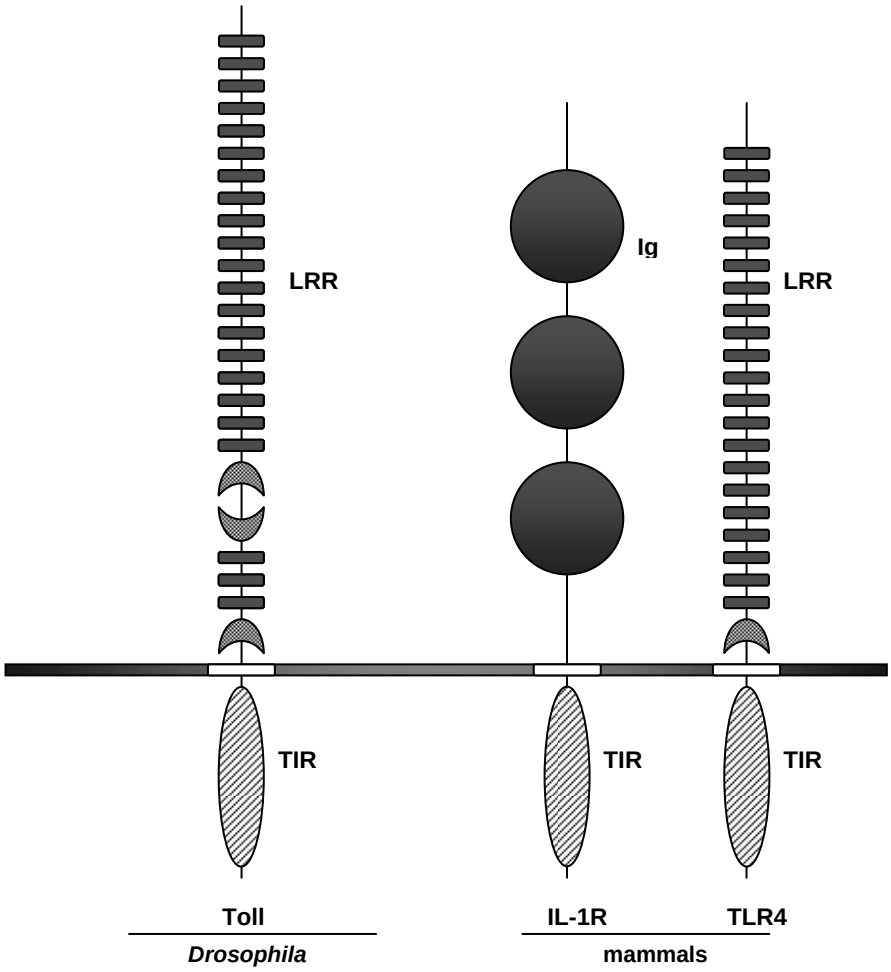

Figure 1.2. ***Drosophila*** **Toll and homologous mammalian receptors**; interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR). The extracellular regions of Toll and TLR4 contain multiple leucine-rich repeats (LRRs, each ▬ representing one such repeat) for ligand binding, while that of IL-1R contains three immunoglobulin (Ig) domains (●). The cytoplasmic domains of all three receptors contain the Toll/interleukin-1 receptor (TIR, ▮) domain for interaction with TIR domain-containing adaptor molecules which activate downstream signaling cascades. The hydrophobic core of the LRR is often capped with cysteine-rich sequences (◠).

1.2.1 *Drosophila* Toll

The discovery of the TLRs was initiated by investigations of Toll. The first member of the Toll family of receptors was identified in *Drosophila melanogaster*, where it was shown to be one of twelve maternal genes involved in embryonic dorsoventral patterning (Hashimoto, Hudson et al. 1988; Belvin and Anderson 1996).

Further investigations indicated that Toll was also important in the control of infections by Gram-positive bacteria and fungi (Imler and Hoffmann 2001). The involvement of this receptor in mediating innate immune responses led to the discovery of sequence homology between the cytoplasmic domains of *Drosophila* Toll and human interleukin-1 receptor and eventually to the discovery of the TLRs in vertebrates (Gay, Packman et al. 1991; Rock, Hardiman et al. 1998). In addition to serving as the stimulus for the discovery of the TLRs, Toll has also been proven to be a valuable model for understanding specific mechanisms of TLR function and as such will be discussed in some detail.

1.2.1.1 Toll Function

The first member of the Toll family of receptors, Toll, was not based on its function as a PRR in innate immune activation. Initially Toll was found to control dorsoventral axis formation in the *Drosophila* embryo. The two primary axes of the *Drosophila* embryo, anterior-posterior and dorsal-ventral, are established by the action of two sets of maternal effect genes (Nusslein-Volhard 1979). Maternal effect genes are maternally-produced and affect the early developmental processes of the embryo. One of the maternal effect dorsal-group genes is *Toll*. Females carrying mutations in the *Toll*

gene produce embryos that have a variety of alterations in their dorsal-ventral pattern (Anderson, Jurgens et al. 1985). Loss-of-function alleles produce dorsalized embryos as a recessive maternal effect, while dominant gain of function alleles result in ventralized embryos. Other recessive alleles cause partial dorsalization or lateralization of the embryonic pattern. Double mutant phenotypes suggest that the products of other dorsal-group genes regulate the activity of *Toll*.

Further investigation of genes in the dorsalization/ventralization pathway indicated that the Toll ligand Spätzle, adapter protein Tube, protein kinase Pelle, transcription factor Dorsal and a Dorsal inhibitor, Cactus, were also involved in the Toll system. Later, the role of Toll in innate immunity was proposed because of noted homology between Toll and the human IL-1 receptor. The intracellular domains of the two receptors are homologous, suggesting conserved function (Janeway Jr. and Medzhitov 2002). It was found that activation of either receptor results in NF-κB activation. Individual receptors within the Toll family, including 18-wheeler and Imd, interact with different combinations of adaptor proteins and activate various transcription factors, including NF-κB, activating protein-1 (AP-1) and interferon regulatory factors (IRFs), driving innate immune responses (Kawai and Akira 2006).

With loss-of-function mutations to *spätzle*, *tube* or *pelle*, fruitflies become highly susceptible to fungal infections (Lemaître, Nicolas et al. 1996), indicating that the Toll pathway controls not only dorsoventral patterning in embryos, but also the antifungal immune response in the adult fly.

1.2.1.2 Toll Structure

Toll is a 135 kDa transmembrane protein with a large extracellular domain and a cytoplasmic domain of 269 amino acids (Winans and Hashimoto 1995) (**Figure 1.3**). The ligand-binding region of Toll contains twenty-two LRRs and carboxy-terminal cysteine-rich domains. The ectodomains of these receptors contain blocks of repeats of a twenty-four amino acid motif, the leucine-rich repeat, usually with a cysteine-rich capping structure at the amino- and carboxy-termini of each block. Toll receptors have two discrete blocks of LRRs and associated capping structures (Chang, Pili-Floury et al. 2004). The LRR units fold into a curved, solenoidal structure, and the sequence conservation reflects the requirements for this structural scaffold, with the conserved residues forming the hydrophobic core of each LRR (Bell, Mullen et al. 2003). The C-terminal capping structure of Toll is connected to a single transmembrane α-helix and the cytoplasmic domain, known as the TIR domain or TIR identity region, which couples downstream signal transduction to receptor engagement. Members of the Toll and TLR families share a conserved molecular structure (Rock, Hardiman et al. 1998).

The basic mechanism of signaling by Toll, and by TLRs, seems to involve ligand-induced dimerization or oligomerization. The evidence for this comes from experiments that examined the interaction of Spätzle with Toll. These studies showed that the binding of Spätzle crosslinks two Toll ectodomains in a symmetrical complex. This crosslinking is necessary and sufficient for signal transduction by Toll (Weber, Tauszig-Delamasure et al. 2003). Recent evidence also indicates that a similar mechanism likely applies to vertebrate TLRs.

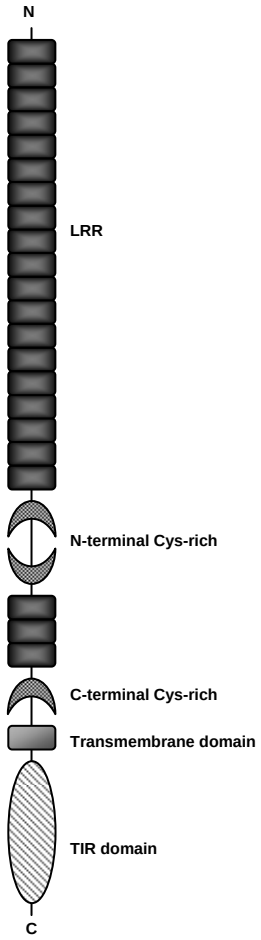

Figure 1.3. ***Drosophila*** **Toll.** The receptor is characterized by a large region of leucine-rich repeats (LRRs, ▬) in the ectodomain, N- and C-terminal cysteine-rich domains (◠), a transmembrane domain (▭) and a cytoplasmic Toll/interleukin-1 receptor (TIR, ▯) domain. The ectodomain is the ligand binding region of the receptor and the cytoplasmic TIR domain, the intracellular signalling domain which interacts with TIR-containing adapter molecules.

Chimeric TLR4 molecules in which the TLR4 ectodomain is replaced with that of Toll are activated by Spätzle with very similar biochemical characteristics to those shown by intact Toll (Weber, Tauszig-Delamasure et al. 2003). This finding indicates that the TIR domains of two chimeric TLR4 molecules can establish downstream signaling if the receptors are arranged in a symmetrical crosslinked complex bound by Spätzle. Though receptor crosslinking is a fundamental event in signal transduction, it is probable that the process of activation involves a series of conformational changes in the receptor (Weber, Moncrieffe et al. 2005).

Studies have shown that the full-length *Drosophila* Toll ectodomain can form unstable dimers in solution in the absence of the ligand Spätzle, although there is no evidence to suggest that the receptor when bound to the membrane is expressed as a stable pre-formed dimer. Conversely, truncations at the N-terminus of the receptor allow the formation of a stable dimeric complex in solution and causes constitutive activation of signaling cascades, indicating that sequences in the N-terminal region of the ectodomain mediate receptor-receptor interactions (Winans and Hashimoto 1995; Medzhitov, Preston-Hurlburt et al. 1997). This also indicates that there is steric hindrance in Toll caused by the N-terminal region of the ectodomain which prevents self-association of the receptors and that binding of Spätzle relieves this constraint. Therefore, the N-terminal region of the Toll ectodomain seems to function as an auto-inhibitor and may provide a structural hindrance that prevents unregulated dimerization of the receptor (Hu, Yagi et al. 2004). The importance of the juxtamembrane region is highlighted by findings showing that point mutations in the cysteine residues of the C-terminal capping structure of the receptor cause constitutive activation of Toll,

indicating that the structural integrity of these structures is required for activation inhibition (Schneider, Hudson et al. 1991). Weber *et al* have demonstrated through deletion mapping experiments of the *Drosophila* Toll ectodomain that the binding sites for Spätzle are located in the N-terminal block of LRRs and that the initial ligand-induced dimerization and the subsequent receptor-receptor interaction are not directly coupled (Weber, Moncrieffe et al. 2005).

The mechanism of Spätzle-Toll binding involves two non-equivalent binding events. The first step, which generates a complex of one ectodomain and one dimeric Spätzle molecule, has a higher binding affinity than the second step, which forms the signaling-competent receptor-ligand-receptor complex (Weber, Moncrieffe et al. 2005). This indicates that the first binding event reduces the affinity for the second binding event, indicating that Toll activation involves negative cooperativity. Negative cooperativity is caused by structural changes in the receptor that are induced in the binding site for the ligand (in this case Spätzle) as a consequence of the first binding event, such that the ligand does not bind as efficiently to the second receptor molecule (**Figure 1.4**).

1.2.2 Discovery of Toll-like Receptors

The involvement of *Drosophila* Toll in mediating innate immune responses ultimately led to the discovery of sequence homology between the cytoplasmic domains of *Drosophila* Toll and human IL-1 receptor (IL-1R) and eventually to the discovery of other Toll-like receptors in vertebrates (Gay, Packman et al. 1991; Rock, Hardiman et al. 1998). The first human homologue of *Drosophila* Toll to be characterized was

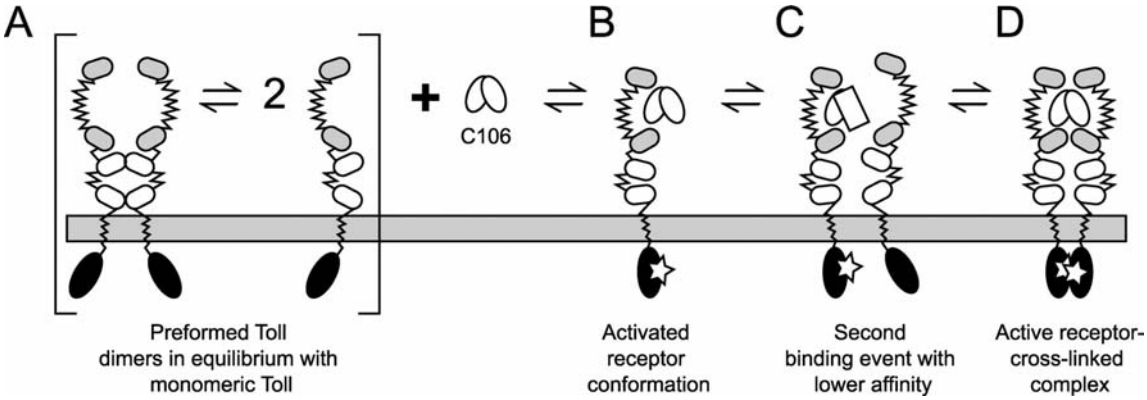

Figure 1.4. Toll activation by Spätzle. Activation of Toll is mediated through a two-step reaction. The first binding event, the formation of a ligand-bound monomer, occurs with high affinity. The second stage of Toll activation, binding of the Toll-ligand monomer by a ligand-free monomer, occurs with lower affinity, representing negative cooperativity of activation. Initially, unbound Toll preformed dimers exist in equilibrium with monomeric Toll. Binding of the ligand Spätzle to one Toll receptor causes the receptor to switch to an active form. Ligand binding reduces the affinity of the second binding event. Another Toll receptor binds the active receptor and a signal is transmitted (Weber, Moncrieffe et al. 2005).

TLR4. It was found that the gene for TLR4 was localized to the same region to which the *Lps* locus (endotoxin unresponsive gene locus) is mapped. Lipopolysaccharide (LPS) plays an important role in sepsis. During sepsis, hyperactive responses result in excessive proinflammatory responses, ultimately leading to tissue injury and cell death. This knowledge led to experiments examining the role of TLR4 in LPS responsiveness in which $TLR4^{-/-}$ mice were created using homologous recombination to replace the transmembrane and cytoplasmic regions of the receptor (Hoshino, Takeuchi et al. 1999). While wild-type macrophage cells responded normally to LPS stimulation with increased levels of tumor necrosis factor α (TNFα) production, $TLR4^{-/-}$ mice showed no TNFα production. Cells from C3H/HeJ mice (LPS-unresponsive) also fail to induce inflammatory cytokines, including TNFα, IL-1 and IL-6 and B cells from these mice do not proliferate in response to LPS exposure. Hoshino and coworkers generated mice lacking TLR4, and found that cells from these mice did not respond to LPS (Hoshino, Takeuchi et al. 1999). Due to the fact that the cells of $TLR4^{-/-}$ mice and LPS-unresponsive C3H/HeJ mice responded similarly, the sequence of the TLR4 gene of C3H/HeJ mice was examined. Sequence comparison between the TLR4 gene of LPS-responsive C3H/HeN mice and that of C3H/HeJ mice showed a point mutation in the cytoplasmic region of TLR4, a proline to histidine mutation at position 712, accounted for the difference in responsiveness to LPS. This was confirmed with NF-κB activation experiments where cells from C3H/HeJ mice stimulated with LPS showed reduced induction of NF-κB (Hoshino, Takeuchi et al. 1999; Arbour, Lorenz et al. 2000). These results demonstrated conclusively that TLR4 was in fact required for responsiveness to

LPS and that defects in TLR4 lead to abnormalities in responses to LPS-mediated immune stimulation.

1.2.2.1 Members of the Toll-like Receptor Family

The TLR family displays diversity between species in regards to expression and function: TLRs 1 through 9 are conserved both in mice and humans, yet TLR10 is present only in humans and TLR11 is functional only in mice. TLR8, though expressed in mice, has been shown to be non-functional. Here a brief description is provided for each of the TLRs and known information for their associated PAMPs.

TLR1, TLR2 and TLR6:

While LPS was originally considered to be the ligand for TLR2, Wetzler and coworkers subsequently demonstrated that contaminating bacterial lipoprotein in the LPS preparations, rather than the LPS itself, resulted in TLR2 activation (Wetzler 2003). A wide range of ligands are now known to bind TLR2, including Gram-positive peptidoglycan (Takeuchi, Hoshino et al. 1999), lipoarabinomannan from mycobacterial species (Means, Wang et al. 1999; Underhill, Ozinsky et al. 1999), *Trypanosoma cruzi* glycosylphosphatidylinositol (Campos, Almeida et al. 2001) and phenol-soluble modulin from *Staphylococcus epidermidis* (Hajjar, O'Mahony et al. 2001).

The ability of TLR2 to recognize such a broad range of ligands results from cooperation with other TLRs, specifically TLR1 (triacyl lipoproteins) or TLR6 (diacyl lipoproteins), through dimerization (Ozinsky, Underhill et al. 2000; Takeuchi and Akira 2001). For example, while the TLR2/TLR1 heterodimer recognizes a wide variety of

lipoproteins, the TLR2/TLR6 heterodimer recognizes only diacylated lipoproteins (Takeuchi and Akira 2001; Takeuchi and Akira 2002; Wetzler 2003). Heterodimerization of TLRs increases the range of PAMPs which are recognized by the receptors.

TLR4:

The first identified TLR, TLR4, remains the best characterized of the TLRs. LPS, the natural ligand for TLR4, activates the innate immune system leading to the production of numerous proinflammatory mediators, such as TNFα, IL-1 and IL-6 (Medzhitov, Preston-Hurlburt et al. 1997; Akira and Takeda 2004).

The recognition of LPS by TLR4 requires several accessory proteins and the formation of a complex between several soluble, membrane-linked molecules, including CD14 and MD-2, and TLR4 (West, Koblansky et al. 2006). LPS is first bound to a serum protein (LPS-binding protein), and the LPS monomer is transferred to CD14, a high-affinity LPS LRR receptor. CD14 is then proposed to transfer LPS to the TLR4-accessory protein complex at the cell membrane (Mathison, Tobias et al. 1992; Ulevitch and Tobias 1999). This demonstrates that, while there may not be a direct interaction between receptor and ligand, accessory proteins play important roles in ligand recognition.

Though LPS is the best-characterized ligand for TLR4, this receptor is also suggested to play a role in the recognition of heat shock protein 60 (hsp60), and lipoteichoic acid (LTA) which is the ligand for TLR2.

TLR5:

TLR5 is involved in recognition of flagellin, which is the principal component of flagella from both Gram-negative and Gram-positive bacteria (Hayashi, Smith et al. 2001). Flagellin is proteinous and does not contain atypical features, such as post-translational modifications, and shares similarities to host proteins. This may potentially eliminate recognition by the innate immune system.

The recognition of bacterial flagellin by TLR5 relies on the conserved, central core structure of flagellin which is necessary for protofilament assembly and bacterial motility (Smith, Andersen-Nissen et al. 2003). This recognition site is proposed to be highly conserved sequences at the amino- and carboxy-termini within the central core of flagellin (Samatey, Imada et al. 2001). A binding site for flagellin on the ectodomain of TLR5 proposed to be located between residues 386-407, as it has been shown that mutants lacking this domain are unable to interact with flagellin *in vitro* (Mizel, West et al. 2003).

TLR3, TLR7, TLR8 and TLR9:

TLRs 3, 7, 8 and 9 are grouped together because they are unified by both having nucleic acid PAMPs as well as an intracellular localization. These PAMPs, both DNA and RNA, are distinguished from other PAMPs in that they more closely resemble host molecules. In contrast to the cell surface-localized TLRs described, this subset of TLRs is localized to intracellular organelles. The intracellular localization of the nucleic acid-activated TLR subfamily is considered to be a safety mechanism to prevent recognition

of self nucleic acids, while maintaining recognition of non-self nucleic acids (Akira, Uematsu et al. 2006).

TLR3:

Cultured cells expressing TLR3 are responsive to purified dsRNA and polyinosinic-polycytidylic acid (poly I:C) whereas $TLR3^{-/-}$ mice display impaired responses to these ligands (Akira and Takeda 2004). Upon activation, TLR3 induces NF-κB activation and IFN regulatory factor (IRF) 3, leading to the production of antiviral molecules such as type I IFN (Alexopoulou, Holt et al. 2001).

Though the recognition of dsRNA by TLR3 suggests a role for the receptor in antiviral immunity, it has been demonstrated that TLR3 is not required for induction of type I IFN during viral infection. Edelmann and his group have demonstrated that $TLR3^{-/-}$ mice display only minor increases in susceptibility to many viral infections (Edelmann, Richardson-Burns et al. 2004), suggesting that other PRRs may be involved in recognition of dsRNA.

TLR7/8:

TLRs 7 and 8 are homologous receptors and recognize PAMPs in a species-specific manner. Murine TLR7 and human TLR8 recognize virally-derived guanosine- or uridine-rich single-stranded RNA (ssRNA) (Heil, Ahmad-Nejad et al. 2003; Diebold, Kaisho et al. 2004). Both receptors are also responsive to synthetic antiviral imidazoquinoline compounds as well as some guanine nucleotide analogues (Heil,

Ahmad-Nejad et al. 2003; Diebold, Kaisho et al. 2004). Though murine TLR8 is expressed in cultured cells, it appears to be non-functional (Akira, Uematsu et al. 2006).

As cells expressing these receptors are responsive to self and non-self ssRNA molecules (Hemmi, Kaisho et al. 2002; Heil, Ahmad-Nejad et al. 2003; Diebold, Kaisho et al. 2004), it is likely that the endosomal localization of these receptors acts as a safety mechanism to reduce the chances of interaction with self nucleic acids. Extracellular RNAses also act as endogenous safeguards against such an event (Akira, Uematsu et al. 2006). Though expression of these receptors confers responsiveness to ssRNA of both host and pathogen origin, it has been demonstrated that RNA of host origin is significantly less stimulatory than bacterial or viral RNA, suggesting a host modification strategy to distinguish self-RNA from non-self RNA (Kariko, Buckstein et al. 2005). The two main differences between 'self' and 'non-self' RNA are the addition of a 5' methyl cap as well as the addition of a 3' poly (A) tail on eukaryotic RNA (Nelson and Cox 2005). It is possible that the host modification strategy to distinguish self from non-self is the modification of the 3' and 5' ends of the RNA, although this has not been proven.

TLR9:

TLR9 is a unique member of the TLR family, in that it is the only receptor which responds to prokaryotic and viral DNA. This recognition is defined by unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides within a particular base context, and it is known that TLR9$^{-/-}$ mice are unresponsive to challenge with CpG-DNA (Hemmi, Takeuchi et al. 2000). In host genetic material CpG-DNA is highly modified by

methylation and suppressed in frequency. This, in addition to the intracellular localization of the receptor, reduces the potential of TLR9 to be activated by endogenous genomic DNA.

Cellular patterns of TLR9 expression differ between species. In humans, TLR9 is expressed primarily in B cells and plasmacytoid dendritic cells (pDCs) of the immune system but reports also suggest that it is expressed in activated neutrophils and pulmonary epithelial cells (Krieg 2006). In contrast, murine TLR9 is expressed in monocytes and myeloid dendritic cells, in addition to B cells and pDCs (Iwasaki and Medzhitov 2004). As a consequence of this difference in TLR9 expression, it is difficult to make predictions regarding TLR9-mediated responses based on extrapolation of results from different species (Krieg 2006). Results obtained from experiments in murine cells, for example, are not necessarily an indicator of what responses may be generated in human cells.

In addition to bacterial DNA, TLR9 is activated by viral DNA sequences containing unmethylated CpG sequences. The genomes of DNA viruses, such as herpes simplex viruses 1 and 2 (HSV1 and HSV2) and murine cytomegalovirus, are rich in hypomethylated CpG motifs. Activation of TLR9 by viral CpG-DNA results in the induction of inflammatory cytokines and type I IFN secretion in a TLR9-dependent manner (Lund, Sato et al. 2003; Krug, Luker et al. 2004; Tabeta, Georgel et al. 2004).

Although much is known about the ligands and signaling pathways of TLRs 1 through 9 and 11, the biological roles of TLRs 10, 12 and 13 remain unclear, as their expression patterns, ligands and modes of signaling have yet to be defined (West, Koblansky et al. 2006).

1.2.3 Structure of Toll-like Receptors

1.2.3.1 Ligand-Binding Domains

TLRs contain extracellular LRRs which are responsible for pathogen recognition. The transmembrane domain, as well as cytoplasmic TIR domains, are required for initiating intracellular signaling (Akira 2003). For all TLRs, the ligand binding domains consists primarily of a repeating pattern of the LRR motif (Bell, Mullen et al. 2003). This motif is present in a large number of eukaryotic proteins of diverse function, typically sharing the unifying characteristic of involvement in recognition and interaction processes (Kobe and Kajava 2001). Indeed the primary function of the LRR motif appears to be in providing an adaptable structural matrix for biomolecular interactions, the versatility of which is best exemplified by the vast and structurally diverse ligands recognized by different TLRs.

The LRRs of the TLR ectodomains contain a motif that consists of a twenty-four-residue repeat; $xL_2xxL_5xL_7xxN_{10}x\varphi xx\varphi xxxxF_{20}xxL_{23}x$, where x represents any amino acid and φ represents necessary hydophobic residues (Ile, Val, Met, Phe). F is a conserved phenylalanine, which is frequently replaced with other hydrophobic residues and N_{10} is a conserved asparagine which is often replaced with cysteine, serine or threonine (Bell, Mullen et al. 2003). The LRR is a structural motif that folds in to a horseshoe structure and is composed of repeating twenty to twenty-nine amino acid stretches that are rich in leucine, a hydrophobic amino acid (Kobe and Kajava 2001). Each repeating unit has β-strand-turn-α-helix structure and the surface between the helices and sheets is the hydrophobic core of the protein, which is tightly packed with leucine residues (Choe, Kelker et al. 2005).

It has been shown that the ectodomains of the receptors are crucial for TLR-ligand interaction. A nucleotide polymorphism in TLR9, P99L in LRR2, is said to affect function of the receptor (Schröeder and Schumann 2005). Truncation studies of TLR5 have shown that the flagellin binding site is located between residues 386 and 407 on LRR14 (Mizel, West et al. 2003). A mutation in TLR4, D299G, inactivates TLR4 and leads to hyporesponsiveness to LPS (Arbour, Lorenz et al. 2000). Thus, it appears as though the ectodomains, containing the LRRs, are important in ligand binging and recognition.

All TLR ectodomains contain non-LRR sequences at their N- and C-termini. The N-terminal residues form a β hairpin, with a disulfide bond connecting the β strands. This type of structure stabilizes the LRR solenoid by capping the hydrophobic core of the first LRR. In contrast to the N-terminal caps, which are variable among TLRs, the C-terminal regions contain a highly conserved consensus sequence which is also stabilized by disulfide linkages (Huizinga, Tsuji et al. 2002).

1.2.3.2 Structural Determination of Toll-like Receptors

To date no structural information is available for TLR9 to offer insights into ligand binding. However, crystallographic determinations have been performed for human TLR3, which has a degree of sequence identity to human TLR9 [25% identity over the whole protein based on a Blast2 sequence alignment of human TLR3 (U88879) and human TLR9 (AF245704), NCBI] and also binds unmethylated pathogenic nucleic acids within acidic endosomal compartments (Bell, Botos et al. 2005; Choe, Kelker et al. 2005). Human TLR3 is activated by double stranded RNA (dsRNA) associated with

viral infection, endogenous cellular mRNA, and sequence-independent small interfering RNAs.

The three dimensional crystal structure of the TLR3 ectodomain, as determined by Choe and coworkers, is a large, horseshoe-shaped assembly consisting of twenty-three LRRs that adopt a right-handed solenoid structure (Choe, Kelker et al. 2005). The concave (inner) surface is formed from twenty-five parallel β strands, twenty-three from LRRs and one each from the N- and C-terminal cap regions that assemble into a highly curved, continuous β sheet that spans 270^{o} of an arc. The convex (outer) surface contains more diverse secondary structure elements, including a variety of different length of loops, four α helices, seven 3_{10} helices, and two β strands.

Fifteen potential N-linked glycosylation sites are located in the ectodomain of TLR3 (Blom, Sicheritz-Ponten et al. 2004). When the locations of the predicted N-glycosylation sites of the TLR3 ectodomain are examined, they are predominantly located on the concave surface of the receptor. This glycosylation pattern is not unique to TLR3, as other members of the TLR family are predicted to be similarly glycosylated (Weber, Morse et al. 2004). This is unexpected because the concave surface of the TLR3 ecotdomain was predicted to be the ligand-binding site, as it has been shown to be for many other LRR-containing proteins. For example, ribonuclease inhibitor, internalin, and platelet glycoprotein-IBα have been shown to preferentially bind ligands in the cavity created by the β-sheet (Bell, Mullen et al. 2003). The combined presence of glycosylation and the predominant negative charge of the concave surface make it an unlikely site for binding dsRNA. Instead, Choe proposes that the putative ligand

binding site for dsRNA is located on the glycosylation-free face of TLR3, the convex side of a dimerized structure (**Figure 1.5**).

In contrast to the model proposed by Choe and Bell, a binding groove for dsRNA within an inner cavity of a TLR3 monomer (Bell, Botos et al. 2005). The overall structure suggested is that of a large curved solenoid. The concave inner surface consists of a large parallel β sheet with each β strand roughly perpendicular to the solenoid axis and linked to the next strand by loop structure. The consensus hydrophobic residues point toward the interior of the molecule, forming a hydrophobic core. Although glycans, which may affect and direct ligand binding by occlusion, were found on the concave surface of the TLR3 ectodomain, two sulfate ions from the crystallization medium were observed to be bound to the concave surface of the TLR3 ectodomain which would suggest that sulfate ions can mimic the phosphate of the nucleotide backbone and provide clues to the potential binding site.

The horseshoe-shaped core provides a large surface area for ligand interaction and recognition. The extensive β sheet on the concave surface of TLR3 ectodomain forms a stable platform upon which loops, insertions, and short helices are anchored. These irregular features are highly conserved among mammalian TLR3 orthologs and apparently evolved over time to facilitate recognition of pathogens that pose a specific threat to the host. The differential glycosylation among the TLRs could control access to specific interaction surfaces. A character of the TLR structure is the large spatial separation of many of the features on the protein core, raising the possibility that each TLR paralog might recognize several different ligands at different sites, thus helping to

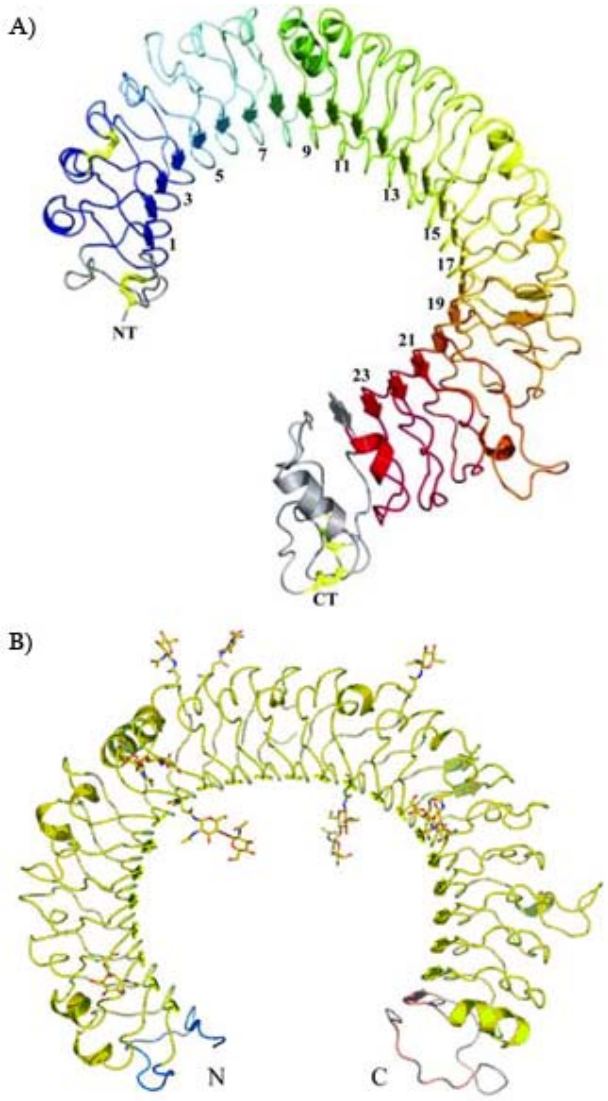

Figure 1.5. Crystal structure of the TLR3 ectodomain. A) Structure of the TLR3 ectodomain as described by Bell *et al*. (Bell, Botos et al. 2005). **B)** Overall ribbon structure of the TLR3 ectodomain as described by Choe *et al*. (Choe, Kelker et al. 2005).

explain the ability of TLRs to recognize a variety of structurally unrelated ligands (Takeda, Kaisho et al. 2003).

Collectively, rather than resolving the structural mechanisms of nucleic acid recognition, these structures add further controversy by proposing two conflicting models of ligand binding; Bell hypothesized a binding groove for double-stranded RNA within an inner cavity of a TLR3 monomer (Bell, Botos et al. 2005) while Choe proposed the convex side of a dimerized structure as the ligand-binding region (Choe, Kelker et al. 2005).

1.2.3.3 TIR Domain

The cytoplasmic portion of TLRs is very similar to that of the interleukin-1 receptor (IL-1R) family. The C-terminal regions of TLRs contain a conserved stretch of approximately 200 amino acids which is required for binding of MyD88 and subsequent signal transmission. This is referred to as the Toll/Interleukin-1 receptor (TIR) domain. Conservation of the TIR domain throughout the TLR family suggests a conserved signaling scheme which would indicate an evolutionarily conserved immune response in insects and vertebrates (Gay and Keith 1991). Indeed, the signaling pathways of the TLRs are homologous to that of the mammalian IL-1R family as a consequence of this conserved cytoplasmic TIR domains found in both receptor families (Takeshita, Gursel et al. 2004).

The TIR domain is required for homotypic protein-protein interactions. A point mutation in the *Tlr4* gene in the TIR domain of TLR4 abolishes responsiveness to LPS, and an equivalent mutation in the TIR domain of TLR2 abolishes responsiveness to

yeast and Gram-positive bacteria (Poltorack, He et al. 1998; Underhill, Ozinsky et al. 1999). Xu and colleagues have elucidated the crystal structures of the TIR domains of human TLRs 1 and 2 (Xu, Tao et al. 2000). The structures reveal that the TIR domain is made up of a central five-stranded parallel β-sheet surrounded by five helices. The core of the TIR domain starts at an (F/Y)DA amino acid motif and ends eight residues C-terminal to a conserved phenylalanine or tryptophan residue, and most of the conserved residues of the TIR domain lie within this hydrophobic core. There are insertions and deletions within the loop regions of TIR domains within the TLRs, which accounts for the differences in size of the TIR domains which range typically from 135 to 160 amino acids. These loop regions contain many conserved residues which are critical for signal transduction, as mutations in these conserved patches have been shown to block signal transduction (Xu, Tao et al. 2000).

Signaling pathways activated via the TIR domain trigger the activation of downstream kinases, and transcription factors such as NF-κB, and the adaptor protein MyD88. MyD88, critical for induction of NF-κB following TLR activation, is a cytoplasmic effector protein which also contains a TIR domain (Bowie and O'Neill 2000). The presence of a C-terminal TIR domain in the effector enables interactions with the TIR domains of the TLRs. As a testament to the central role that MyD88 plays in MyD88-dependent TLR-mediated innate immune response, MyD88-deficient mice do not respond to peptidoglycans, flagellin, CpG-DNA or ssRNA (Adachi, Kawai et al. 1998; Takeda and Akira 2003; Beutler 2005; Yarovinsky, Kanzler et al. 2006).

1.2.3.4 Pathways Activated

Following activation of a Toll-like receptor by its PAMP several outcomes are possible. Cellular responses are observed as immune cells produce signaling factors, chemokines and cytokines, which trigger inflammation. In the case of a bacterial factor, the pathogen might be phagocytosed and digested, and its antigens presented to CD4+ T cells. In the case of a viral pathogen, the infected cell may shut off its protein synthesis and may undergo programmed cell death (apoptosis). Immune cells that have detected a virus may also release anti-viral factors such as interferons which inhibit viral multiplication and modify the body's immune response. The discovery of the TLRs finally identified the innate immune receptors that were responsible for many of the innate immune functions that had been studied for many years. Interestingly, TLRs seem only to be involved in the cytokine production and cellular activation in response to microbes, and do not play a significant role in the adhesion and phagocytosis of microorganisms.

MyD88 is the functional homolog of *Drosophila* Tube in that it is an adaptor protein. Like Tube, MyD88 has a death domain involved in recruitment of the downstream serine/threonine kinase (Pelle for Tube, IRAK for MyD88), whereas, in contrast to Tube, it has a TIR domain, most likely involved with interactions with IL-1R/TLR family receptor complexes. MyD88 has recently been confirmed as having a central role in the immune signaling cascades of the IL-1R/TLR family in that $MyD88^{-/-}$ mice, the responses to IL-1, IL-18 and LPS are all abrogated (Adachi, Kawai et al. 1998; Kawai, Adachi et al. 1999).

When activated, TLRs recruit adaptor molecules within the cytoplasm of cells to propagate a signal. Four adaptor molecules are known to be involved in signaling. These proteins are MyD88, TIR domain-containing adaptor protein (Tirap, also called Mal), TIR domain-containing adaptor protein inducing IFN-β (Trif) and Trif-related adaptor molecule (Tram) (Yamamoto, Sato et al. 2002; Yamamoto, Sato et al. 2003; Yamamoto, Sato et al. 2003). The adaptors activate other molecules within the cell, including certain protein kinases (IRAK1, IRAK4, TBK1 and IKKi) that amplify the signal, and ultimately lead to the induction or suppression of genes that orchestrate the inflammatory response. In all, thousands of genes are activated by TLR signaling and, collectively, the TLR family constitutes one of the most powerful and important gateways for the modulation of gene expression (**Figure 1.6**).

Understanding of the kinases involved in NF-κB activation has increased in recent years. These kinases, IκB kinase (IKK) α and β, exist as part of a larger IKK signalsome that contains other scaffold and regulatory proteins. IKKα and β form a core IKK complex together with IKKγ (or NEMO, NF-κB essential modulator), which seems to be the minimal requirement for NF-κB activation (Bowie and O'Neill 2000). Many upstream kinases have been shown to be capable of activating the IKK complex *in vitro*, including NIK, MEKK1, TAK1, protein kinase Cζ, and MEKK2 (Lee, Hagler et al. 1997; Zhao and Lee 1999), which may explain how diverse stimulants can activate NF-κB. In terms of IL-1R/TLR pathways, MEKK1 and NIK are particularly important (Muzio, Ni et al. 1997; Kopp, Medzhitov et al. 1999). Although the different IL-1R/TLR family members use similar signaling pathways, there may be subtle differences in the adaptor proteins recruited during signaling.

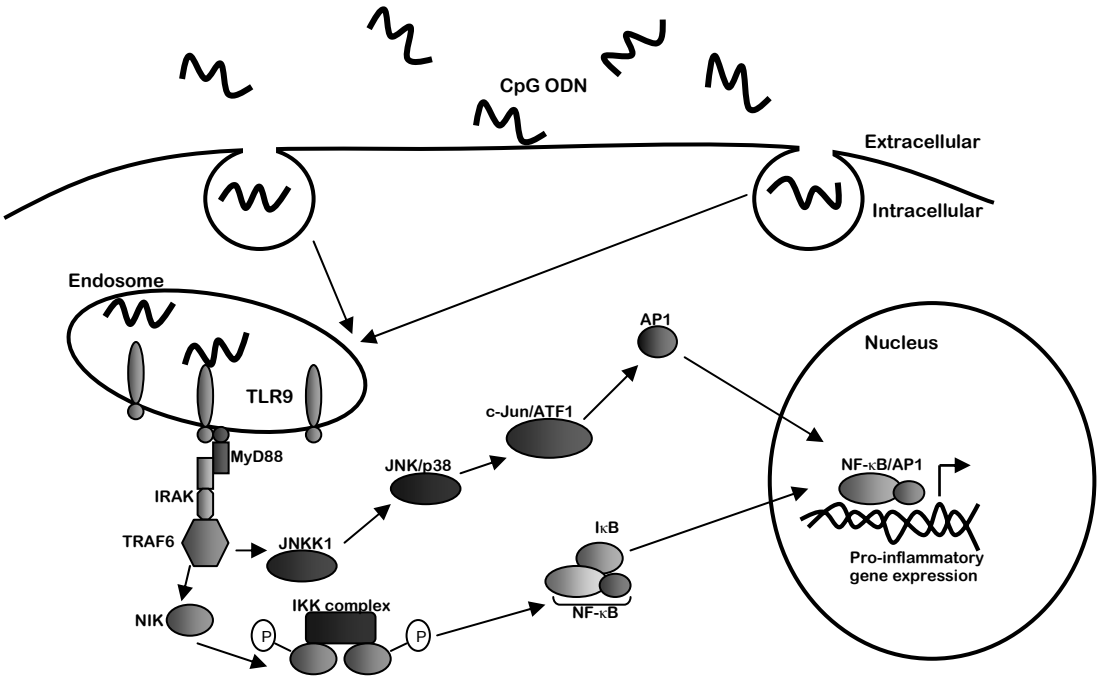

Figure 1.6. Signal transduction associated with TLR9. Binding of bacterial DNA or CpG ODNs promotes activation of TLR9 through dimerization. The ligand-bound dimer recruits MyD88 to the membrane and initiates association with adaptor molecules such as IRAK4. MyD88 activates IRAK4 to phosphorylate IRAK1. Once phosphorylated, IRAK1 recruits TRAF6 to activate TAK1, resulting in activation of the MAPK signaling cascades, JNK1 and p38. TRAF6 also activates the IKKs, which phosphorylate IκB. Phosphorylated IκB dissociates from NF-κB and is degraded. NF-κB translocates to the nucleus to induce expression of proinflammatory cytokines. The resulting pattern of gene expression induces several immunomodulator effects including cell-mediated immunity, apoptosis as well as direct antimicrobial activity.

All TLRs elicit conserved inflammatory pathways, culminating in the activation of NF-κB and AP-1. NF-κB is a dimeric transcription factor that belongs to the Rel-homology domain-containing protein family, which includes p65/RelA, p50/NF-κB1, p52/NF-κB2, RelB and c-Rel (Karin and Greten 2005). TLR signaling leads to activation of several transcription factors, including NF-κB and IRFs, which subsequently mediate the induced expression of a variety of immune response genes. Each TLR activates similar signaling pathways, but some TLRs trigger specific and unique pathways. This differential induction pattern depends heavily on cytoplasmic adaptor molecules that can associate with the intracellular region of TLRs (Akira 2003).

1.3 Toll-like Receptor 9

1.3.1 Discovery of TLR9

The discovery of Toll in *Drosophila* lead to the discovery of the TLR family. After the characterization of the first mammalian TLR, TLR4, several proteins related to TLR4 were identified and named TLRs (Rock, Hardiman et al. 1998). Prior to a study conducted by Hemmi, the ligand for TLR9 was unknown and the receptor had not yet been characterized. At the time only TLRs 1-6 had been identified, and the sequences for TLRs 7 and 8 deposited in to GenBank. Using a BLAST search, Hemm and coworkers identified an expressed sequence tag (EST) which had high degree of similarity to the previously identified TLRs. Using the EST as a probe, a full-length complementary DNA (cDNA) was isolated from the murine and human cDNA libraries. Sequence analysis indicated that these cDNAs contained regions conserved within the TLR family, including the LRR and TIR domains. The gene was designated *Tlr9*.

To determine the biological function of TLR9, analyses of TLR9-deficient ($TLR9^{-/-}$) mice were carried out and revealed that TLR9 is a receptor for a cytosine-phosphate-guanine (CpG) motif in DNA (Hemmi, Takeuchi et al. 2000) as $TLR9^{-/-}$ mice did not show any response to CpG DNA, including cell proliferation and production of inflammatory cytokines. In addition to bacterial CpG DNA, TLR9 has been shown to recognize viral-derived CpG DNA in plasmacytoid dendritic cells (Lund, Sato et al. 2003; Krug, Luker et al. 2004). TLR9-mutant mice have also been shown to be susceptible to murine cytomegalovirus (MCMV) infection (Tabeta, Georgel et al. 2004), indicating that TLR9 is implicated in the recognition of this virus.

1.3.2 Bacterial DNA as a PAMP

While attempting to identify the antitumor components of Bacillus Calmette-Guerin extracts, Tokunaga and colleagues established that bacterial genomic DNA may account for the observed immune stimulation (Tokunaga, Yamamoto et al. 1984). Messina later demonstrated that poly-(dC, dG) from purified bacterial DNA was able to induce B cell proliferation, while vertebrate DNA did not have this activity (Messina, Gilkeson et al. 1991). In a subsequent report by Krieg, it was reported that the mitogenic activity of bacterial DNA to B cells was due to the presence of unmethylated CpG dinucleotides in a particular base sequence termed the CpG motif (Krieg, Yi et al. 1995). In agreement with previous studies, methylation of CpG sequences removed the immunostimulatory activity.

Different genera of bacteria have differences in the C/G content of their DNA. Since TLR9 recognizes CG-containing DNA motifs, it can be assumed that DNA from

different bacteria should vary in their capacity to activate TLR9. Dalpke *et al* observed that bacterial DNA from different species do, in fact, differ in their potential to stimulate TLR9 (Dalpke, Frank et al. 2006) and that the CG content, that is to say the DNA sequence, of the bacterial DNA had an effect on the activity of TLR9.

1.3.2.1 CpG Motifs

Foreign and host DNA appear to be discriminated on the basis of unmethylated CpG dinucleotide sequences which are frequent within microbial DNA but largely absent from host cell genetic material (Bird 1986; Krieg, Yi et al. 1995; Krieg 2002). The immunostimulatory activity of the CpG motif is found in non-self DNA, while mammalian cells ignore high concentrations of their own (self) DNA. Bacterial DNA contains unmethylated CpG motifs, which give the DNA its immunostimulatory activity. In vertebrates, the frequency of CpG motifs is greatly reduced, approximately 1/5 of the frequency observed in bacterial DNA, and the cytosine residues of the motifs are highly methylated, decreasing, or eliminating, the immunostimulatory activity (Bird 1986; Deng, Nilsson et al. 1999; Takeda and Akira 2005). CpG DNA has been shown to be an excellent immune adjuvant in various models of murine disease (Weiner, Liu et al. 1997; Davis, Weeranta et al. 1998). CpG-induced activation of innate immune responses protects against lethal challenge with a variety of pathogens, and has therapeutic activity in murine models of cancer and allergy (Krieg 2002). CpG ODNs also enhance the development of acquired immune responses for prophylactic and therapeutic vaccination.

1.3.2.2 Higher Order Species-Specific Activation

In addition to the CpG dinucleotide, TLRs also recognize higher order sequences around the dinucleotide. These sequences appear to be recognized in a species-specific fashion. Mouse TLR9 is most responsive when the CpG motif is flanked by purine residues on the 5' side and pyrimidine residues on the 3' side; GACGTT (Yu, Kandimalla et al. 2003); human TLR9 by GTCGTT or TTCGTT sequences (Bauer, Kirschning et al. 2001). Therefore, the specificity of TLR9-dependent activation of the immune system may involve recognition of higher order motifs beyond the CpG dinucleotide.

Responsiveness to immunostimulatory CpG DNA correlated with human TLR9 expression in human immune cells (Bauer, Kirschning et al. 2001). Expression of TLR9 in human non-responder cells results in these cells becoming responsive to immunostimulatory CpG DNA, indicating that the specificity represents preferential binding by TLR9. Transfection of either human or murine TLR9 conferred responsiveness, yet required species-specific CpG DNA motifs for initiation of signaling pathways (Bauer, Kirschning et al. 2001). Non-optimal ODNs gave delayed and less sustained activation of signaling pathways (Roberts, Sweet et al. 2005). When the CpG dinucleotide is inverted to GC in the ODNs, some residual activity was retained in a species-specific, TLR9-dependent manner (Rutz, Metzger et al. 2004).

1.3.3 TLR Localization

1.3.3.1 Intracellular Localization of Nucleic Acid-binding TLRs

TLR3, TLR7, TLR8 and TLR9 have evolved to recognize the nucleic acids of bacteria and viruses. However, nucleic acids are also endogenous to mammalian cells, and it is important that these molecules not be recognized as "non-self" to avoid the activation of inflammatory responses. A way that "self"-nucleic acids may be prevented from activating TLRs is through their physical separation from the nucleic acid-binding TLRs. The ligand-binding domains of TLRs are situated either on the cell surface or in intracellular compartments. Nucleic acid-binding TLRs are located intracellularly, rather than at the cell surface. The sequestration of the nucleic acid-binding TLRs to intracellular locations may reflect a strategy to avoid inappropriate activation by self molecules. Interestingly, the ability of these receptors to bind their ligands may require non-neutral pHs, such as the acidic environment that exists within the endosomes (Watts 2004; Bell, Askins et al. 2006; Gibbard, Morley et al. 2006).

Studies have shown that phagocytosed antigens do not enter the appropriate intracellular location in the absence of TLR ligands, indicating that cells of the immune system that are not activated by TLRs do not process and present antigen (Blander and Medzhitov 2004). This also suggests that phagosomal maturation is regulated by TLRs. Co-localization of the TLR signal and the antigen may be a crucial factor. Another study focused on recognition of cytosolic nucleic acids, showing that while wild-type receptors were not activated by host nucleic acids, chimeric receptors improperly localized at the cell surface, rather than in intracellular compartments, were responsive to host nucleic acids (Heil, Ahmad-Nejad et al. 2003; Barton, Kagan et al. 2006).

TLRs 3, 7, 8 and 9 are all closely related in sequence (**Figure 1.7**) and have the same conserved features, such as a cysteine-rich insert in LRR8 (Bell, Mullen et al. 2003; Matsushima, Tanaka et al. 2007), indicating that they might have related mechanisms for the recognition of nucleic acids and nucleotide analogues. These TLRs are localized to intracellular compartments rather than at the cell surface, and it has been demonstrated that this subcellular localization is required for their function (Lee, Chuang et al. 2003; Latz, Schoenemeyer et al. 2004). Medzhitov showed that the subcellular localization is important for specificity and for the discrimination of self from non-self nucleic acids (Medzhitov and Janeway Jr. 2000; Barton, Kagan et al. 2006). They showed that the intracellular localization of TLR9 is specified by the transmembrane α-helix and that a chimeric receptor composed of the TLR9 ectodomain and the TLR4 transmembrane and cytoplasmic domains is trafficked to the cell surface, indicating that the transmembrane domain is required for localization. These hybrids are able to signal in response to exogenous self nucleic acids, whereas native TLR9 responds only to viral or bacterial CpG-containing DNA.

1.3.3.2 Trafficking and Requirement for Acidification

Many experiments have been performed to determine whether CpG DNA moves to the same compartment in which TLR9 is expressed, or whether TLR9 moves to where the ligand, CpG DNA, is located (Latz, Schoenemeyer et al. 2004; Yasuda, Yu et al. 2005). It has been well-established that unmethylated CpG-containing plasmids induce activation of NF-κB in a TLR9-dependent manner (Bauer, Kirschning et al. 2001). This activation is highly specific because methylated CpG-containing plasmids,

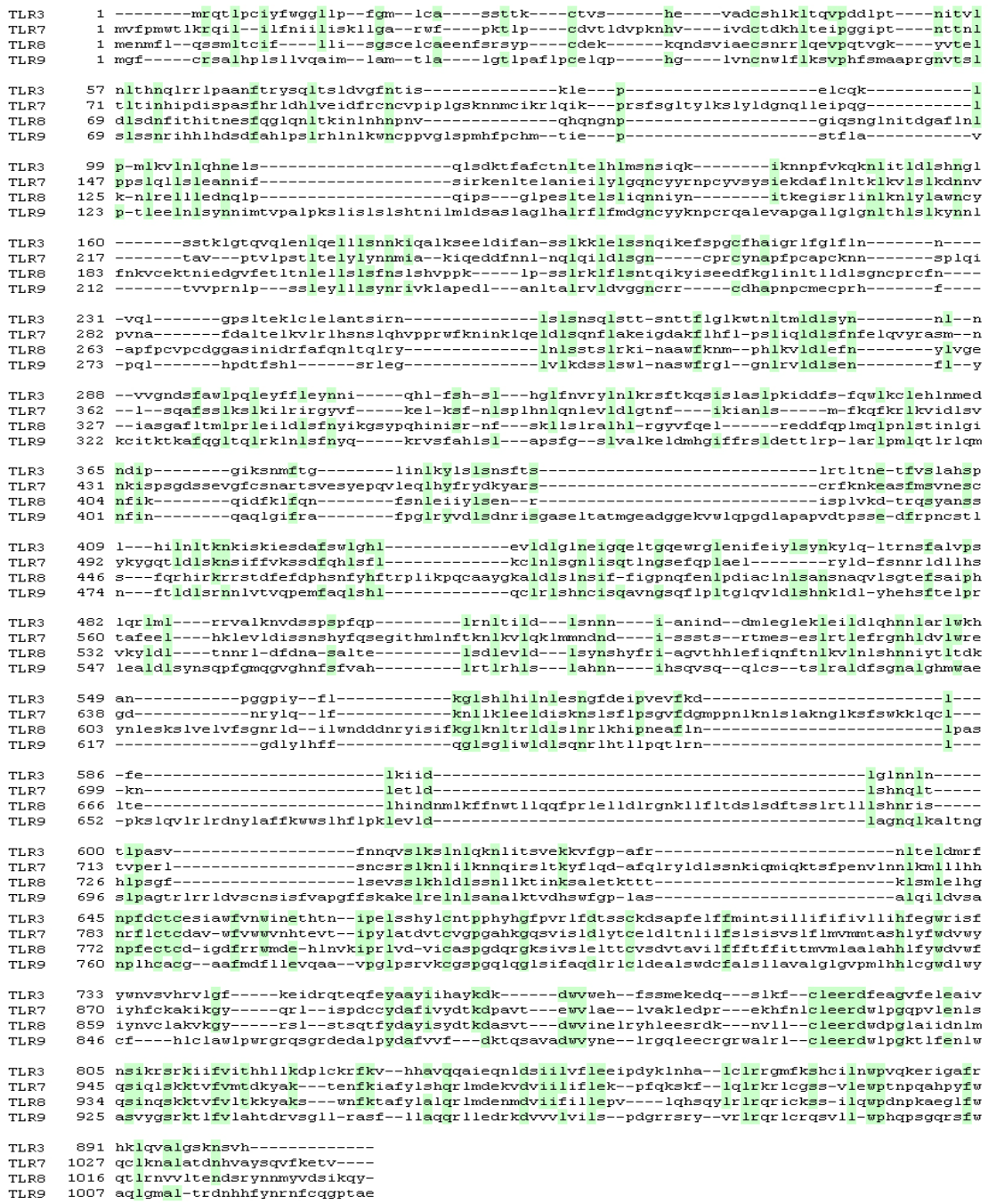

Figure 1.7. Protein sequence alignment of human TLRs 3, 7, 8 and 9. Colored regions represent exact base matches. Using TLR3 (GenBank ascension number U88879) as a reference: TLR7 (AF240467) has 26% exact base matches, TLR8 (AF245703) has 26% and TLR9 (AF245704) has 24% exact base matches.

as well as methylated CpG-ODNs, are ineffective in activating NF-κB. It is also established that chloroquine, an agent which blocks endosomal maturation, blocks, at least in part, cellular activation by the unmethylated CpG-containing plasmid or by CpG-ODN, suggesting that endosomal maturation may be required for TLR9 signaling (Hartmann and Krieg 1999). The LPS transduction pathway via TLR4, which can use the same adaptor proteins as TLR9, was not inhibited by chloroquine and derived compounds (Yi, Tuetken et al. 1998), which suggests that the activation of the signal transduction pathway induced by plasmid DNA requires its internalization and the acidification of the endosome.

It has been established that in the resting cell, most of the cellular TLR9 resides in the endoplasmic reticulum (ER) (Leifer, Kennedy et al. 2004). How TLR9 gains access to internalized CpG ligands is not well-understood, but it is known that TLR9 can be detected in lysosomes after stimulation with CpG DNA (Latz, Schoenemeyer et al. 2004). It has been shown that TLR9 is retained in the ER prior to CpG DNA exposure (Latz, Schoenemeyer et al. 2004; Leifer, Kennedy et al. 2004), and it has been proposed that TLR9 gains access to endosomes containing CpG DNA by direct fusion of the endosome with the ER.

The fact that all of the intracellular TLRs identified share specificity for nucleic acids suggests that this localization is related to the recognition of this class of ligands. Nucleic acid ligands may need to be modified in acidified compartments before they can be recognized. Also, intracellular compartments may serve to concentrate ligands in such a way as to reach the threshold required to activate the receptor. The degradative environment of mature endosomes and lysosomes may break down bacteria or viruses,

releasing nucleic acids for the recognition by TLRs. Finally, the intracellular localization of TLRs 3, 7, 8 and 9 may help distinguish the sources of nucleic acids. This arises from the issue that, in contrast to most TLR ligands, nucleic acids can be either foreign or self (Leadbetter, Rifkin et al. 2002; Viglianti, Lau et al. 2003). Mammalian DNA, for example, can stimulate TLR9 in some situations (Barton, Kagan et al. 2006). Therefore, a mechanism must exist to ensure that TLRs involved in nucleic acid recognition can discriminate between foreign and self nucleic acid.

1.3.4 ODNs as TLR9 Ligands

1.3.4.1 ODN Classes and Actions

The immunostimulatory action of bacterial DNA can be mimicked with short single-stranded oligodeoxynucleotides (ODNs). These ODNs have considerable advantages as therapeutic TLR9 agonists as a result of their defined sequences and structures, low cost and chemical stability. To achieve greater biological stability through nuclease resistance, the majority of TLR9 investigations utilize phosphorothioate-modified (PTO) ODNs in which one of the non-bridging backbone oxygen atoms is replaced with sulfur (**Figure 1.8**)

At least three distinct classes of oligonucleotides containing CpG motifs have been reported based on their ability to induce different cellular responses (Klinman 2004) (**Table 1.4**). Conventional CpG DNA, B-type CpG DNA, has the potent ability to activate B cells and induce cytokine production from macrophages. B-type CpG DNA has multiple CpG motifs, and stabilized by a phosphorothioate backbone (Krieg 2002). A-type CpG DNA shows a weaker ability to activate B cells or macrophages but

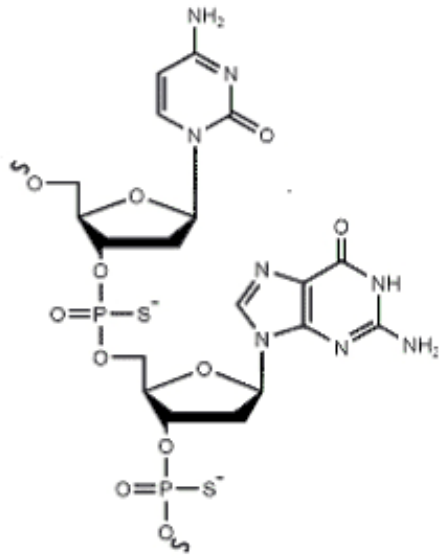

Figure 1.8. Chemical structure of a phosphorothioate CpG ODN. A non-bridging oxygen atom is replaced with a sulfur atom to differentiate phosphodiester ODNs from the modified form (Integrated DNA Technologies, www.idtdna.com).

Table 1.4. Representative sequences, structures and biological actions of the different ODN classes. Adapted from Klinman, DM. 2004 (Klinman 2004).

ODN Type	Example	Structural characteristics	Associated activity
A	GG**TGCAT*CG*ATGCA**GGGGGG	PD and PTO backbone Single *CpG* motif Poly G tail **Hairpin** forming sequences	Stimulate pDCs to release IFN-α IFN-α-mediated APC maturation
B	TCCATGGA*CG*TTCCTGAG*CG*TT	PTO backbone Multiple *CpG* motifs	Maturation of pDC and TNF production B cell proliferation IL-6 production
C	T**CGTCGTT*CG*AACGA*CG***TTGAT	PTO backbone Multiple *CpG* motifs **Hairpin** forming sequences	Stimulate pDCs to release IFN-α B cell proliferation IL-6 production

a stronger ability to induce type I IFN production from plasmacytoid dendritic cells. A-type CpG DNA contains only a single CpG motif, and the activity requires a mixed backbone of phosphodiester-phosphorothioate (Krug, Rothenfusser et al. 2001). In addition, the region around a CpG motif should be palindromic and phosphodiester-linked, and a poly-G tail should be included at the 3' end. C-type CpG DNA exhibits type I IFN-inducing, as well as B cell-activating, functions. It is comprised of a phosphorothioate backbone with multiple CpG motifs and a TCG dimer at the 5' end (Marshall, Fearon et al. 2003). B-type CpG DNA is rapidly transferred and degraded in the lysosome, whereas A-type CpG DNA is retained in the endosome of pDCs, together with signal-transducing molecules, such as MyD88 and IFN regulatory factor (IRF) 7 for long periods. If B-type CpG DNA is modified by cationic lipids to be retained in the endosome, it can induce type I IFN production, even from conventional DCs. Through G tetrad formation of poly-G tail, A-type CpG DNA tends to self-assemble in to a nanoparticle, which resembles the viral structure (Kerkmann, Costa et al. 2005). This particular structure contributes to intracellular localization of A-type CpG DNA. Structurally, the three classes of ODNs differ in the number and positioning of CpG motifs, as well as in the presence of backbone modification and the extent to which the backbone is modified (Krieg 2002). When these ODN sequences were subject to computational secondary structure prediction analysis, it was observed that many of the ODNs have the potential to adopt higher order structures based upon the formation of hairpin structures (Verthelyi, Ishii et al. 2001). It is postulated that the observed ODN class-specific differences in immunostimulation may be dictated by the different fit between each class of ODN and the TLR9 molecule. For instance, the binding of each

class of CpG ODN and the TLR9 molecule may trigger different conformational changes in the receptor and/or promote recruitment of unique co-signaling adaptor molecules or accessory proteins. Class-specific responses suggest that although all CpG ODNs can interact with TLR9, there are likely differences in the mechanisms of trafficking, recognition and/or signal transduction induced by each class either due to the sequence/structure differences between the ODNs or differences in accessory proteins or co-stimulatory molecules that are recruited to the TLR9-ODN complex which have not yet been identified.

1.3.4.2 Functional Consequences of the PTO Modification

PD-ODNs are inherently susceptible to nucleases and as such are rather unstable. In order to achieve greater biological stability through nuclease resistance, the majority of TLR9 investigations utilize PTO-ODNs. These ODNs are known to have CpG-independent side effects including induction of B cell proliferation, splenomegaly, and tissue infiltration by mononuclear cells, indicating that the PTO-modified backbone does behave independently of CpG, and behaves differently than the unmodified PD backbone (Barton, Kagan et al. 2006; Yasuda, Rutz et al. 2006). While PD-ODNs are unstable and have poor cellular permeability, PTO-ODNs are more stable and are taken up more efficiently by cells.

The PTO modification is not a neutral substitution, although it is not yet clear how this modification influences the specificity of interaction with TLR9. PTO-ODNs have been described as “sticky” with the tendency to interact with other proteins, including other TLRs (Rutz, Metzger et al. 2004). The interactions with proteins other

than TLR9 may account for the ability of PTO-ODNs to exert CpG-independent side effects (Khaled, Benimetskaya et al. 1996; Baek, Ha et al. 1997; Monteith, Henry et al. 1997). Clearly, TLR9 activation by PTO-ODNs and natural PD-ODNs does in fact differ (Kirk and Bazan 2005).

1.3.5 TLR9 as a Therapeutic Target

1.3.5.1 Uses and Advantages

The low cost, ease of production and stability of nucleic-acids ligands make TLR9 an attractive target for immunotherapeutic intervention. These nucleic acid ligands, particularly CpG-ODNs, have the additional advantage of being one of the most selective stimulators of dendritic cells with minimal systemic toxicity (Ioannou, Griebel et al. 2003). The ability for CpG treatment to modulate innate immune responses has given rise to a number of therapeutic applications including: 1) priming the innate immune system to mediate host protection, 2) as adjuvants to promote induction of antigen-specific immune responses, 3) as anti-allergens through establishment of T_H1 responses, 4) in the treatment of a variety of malignancies and 5) for improved vaccination efficiency of individuals with poor immune responses (Krieg, Yi et al. 1999; Dittmer and Olbrich 2003; Klinman 2004; Lazarczyk, Grzela et al. 2005).

Therapeutic targeting could be done by: 1) prevention of ligand binding to LRR domains of receptors; 2) blocking the interactions between receptors and adaptors in the signaling pathways; 3) blocking the enzymes in signaling pathways and; 4) immunostimulation with vaccine adjuvants. There are different signaling events which occur after receptor-ligand interaction and it is theoretically possible to design new

therapeutics for human immune diseases and inflammatory disorders using knowledge of the signaling pathways. The information regarding the structure of TLR9 and of pathways activated could also be used in therapeutic approaches for modulating the innate immunity. In addition to PAMPs, synthetic analogues of PAMPs and fully synthetic small molecules could be used as agonist or antagonist ligands of the TLRs.

1.3.5.2 Potential Problems

Though CpG DNA is one of the most selective stimulators of dendritic cells with minimal toxicity, concerns have been raised over the potential for the therapeutic application to trigger the development of autoimmune disease. Studies have shown that high doses of bacterial DNA elicit the production of auto-antibodies against double-stranded DNA in normal mice and accelerate the production of autoimmune antibodies in lupus-prone animals (Gilkeson, Ruiz et al. 1993). CpG-ODNs may also facilitate the development of toxic shock by lowering the pathological threshold of LPS tolerance (Sparwasser, Miethke et al. 1997). It has also been demonstrated that ODNs can exhibit sequence-independent effects, indicating that the stimulatory capacity of the ODN is due to more than the DNA sequence. While the risks associated with these scenarios are low, they emphasize the importance of optimization of TLR9 agonists.

1.3.6 Current Status of Knowledge

1.3.6.1 Binding of Double-stranded Nucleic Acids

It has been demonstrated that innate immune responses can be initiated by plasmid DNA (Sato, Roman et al. 1996; Roman, Martin-Orozco et al. 1997) and time

course experiments verify that plasmids remain intact within the endosomes for sufficient time to serve as TLR9 ligands (Bennett, Gabor et al. 1985). Previous investigations have reached opposing conclusions, however, with respect to the ability of TLR9 to bind double-stranded DNA. While a system established by Cornélie demonstrated effective binding of plasmids to TLR9 (Cornélie, Hoebke et al. 2004), an independent investigation by Rutz *et al* reported only a weak interaction between TLR9 and double-stranded ligands, indicating that the immunostimulatory activity of the CpG DNA was dependent on the single-stranded character of the molecule (Rutz, Metzger et al. 2004).

Surface plasmon resonance (SPR) has been used to demonstrate the binding of CpG DNA by TLR9. Cornélie and his group were able to show that murine TLR9 binds unmethylated CpG-containing plasmid. The interaction was shown to be sequence-specific, influenced by the methylation status of the plasmid and lead to activation of the NF-κB pathway in TLR9-expressing cells (Cornélie, Hoebke et al. 2004).

SPR was primarily used to study the physical interaction between murine TLR9 and a CpG-containing plasmid. When unmethylated CpG-containing plasmid was injected on to the sensor chip, the absorption signal was increased. This signal was enhanced with increasing doses of plasmid, indicating that the unmethylated CpG-containing plasmid was binding to murine TLR9. In contrast, methylated CpG-containing plasmid showed only a marginal increase in signal absorption which was not enhanced with increasing doses of plasmid, indicating that the binding was non-specific (Cornélie, Hoebke et al. 2004).

1.3.6.2 Sequence Specificity

While cell stimulation assays suggest the preferential ability of CpG ODNs to initiate cellular responses, *in vitro* binding assays have reached contrasting conclusions with respect to the ability of TLR9 to bind nucleic acids in a sequence-specific fashion.

Using SPR to prove that the receptor-ligand interactions were CpG-specific competition experiments were performed with plasmids, CpG-ODNs and GpC-ODNs. CpG-ODNs were able to inhibit binding of the plasmid to murine TLR9, while the inverted GpC-ODN did not inhibit binding of the plasmid. This indicated that murine TLR9 was in fact the receptor for plasmid DNA and that binding of DNA to the receptor was dependent on the presence of unmethylated CpG motifs (Cornélie, Hoebke et al. 2004).

The results generated with SPR were confirmed with NF-κB activation experiments. Cells were transfected with murine TLR9 as well as a reporter plasmid driven by the NF-κB promoter. Cells were incubated with CpG-ODN or inverted GpC-ODN. CpG-ODN dramatically induced activation of NF-κB, whereas the GpC-ODN induced activity comparable to that detected when mTLR9 was not expressed. This induction was also dependent on the methylation status of the CpG motif. Unmethylated CpG-ODN activated NF-κB, while methylated CpG-ODN did not (Cornélie, Hoebke et al. 2004).

Rutz also used SPR to demonstrate that TLR9 interacts directly and sequence-specifically with CpG-DNA (Rutz, Metzger et al. 2004). They also showed that TLR9-CpG-DNA interaction is blocked by chloroquine and quinacrine, suggesting that both compounds act as TLR9 agonists. In order to further study the molecular interaction

between TLR9 and CpG-DNA, immunoprecipitation experiments were performed. Fluorescently-labeled TLR9 as well as TLR4 were incubated with biotinylated CpG-DNA. CpG-DNA was able to precipitate TLR9, but not TLR4, indicating that the interaction between TLR9 and CpG-DNA was specific. The binding of biotinylated CpG-DNA was out-competed by unlabeled CpG-DNA, further demonstrating specificity of binding (Latz, Visintin et al. 2004). Interestingly, GpC-DNA was also able to precipitate TLR9, and not TLR4, indicating that TLR9 is able to bind ODNs in a sequence-independent manner.

Many studies have also been undertaken to increase uptake of DNA into cells, and to observe whether this would have any effect on activation of TLR9. Use of the liposomal transfection reagent *N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propan methylsulfate (DOTAP) results in a more efficient delivery of DNA into the cell, and this has been shown to be coupled with increased TLR9 activation (Dalpke, Frank et al. 2006). Both the stimulatory and the non-stimulatory forms of DNA can cause this effect (Gibbard, Morley et al. 2006). DOTAP-mediated endosomal translocation of otherwise non-stimulatory vertebrate DNA or of certain non-canonical CpG motifs triggers dendritic cell activation in terms of cytokine production, such as type I IFN and IL-6. The stimulatory activity of PTO-ODNs is TLR9 dependent, whereas phosphodiester DNA, such as vertebrate DNA, in addition trigger TLR9-independent pathways. It has been proposed that the inefficiency of the natural route for DNA internalization prevents low affinity TLR9 ligands in endosomes from reaching threshold concentrations required for TLR9 activation.

1.3.6.3 Current Limitations to the Design of TLR9 Agonists

The design of therapeutic TLR9 agonists has been slowed by the lack of information available for receptor-ligand interaction, as well as the absence of a convenient screening assay. This is largely due to the difficulties inherent in purifying sufficient quantities of the receptor for biochemical studies. Rather than being evaluated on their capacity to interact with the receptor, TLR9 ligands are evaluated on the basis of their ability to elicit cellular responses in immune cells. This approach is limited in that it does not allow discrimination of ligand-binding events from the entire signaling process. To address these issues, we wanted to develop a system that would allow us to purify sufficient functional ligand-binding TLR9 for ligand-binding experiments. We required a system that would produce significant amounts of glycosylated, active and reasonably pure protein. In this thesis, a heat shock system was investigated as a potential mechanism to overcome the problems with purifying active receptor (Kowalski, Gilbert et al. 1993). This inducible, non-destructive system allows high levels of expression of foreign genes and protein products.

2.0 HYPOTHESIS AND OBJECTIVES

Using an established heat shock system for expression of the ligand binding domain of human TLR9, sufficient quantities of active receptor will be produced for biochemical assays to characterize the ligand binding domain and gain insight in to ligand binding characteristics.

Objectives:

1. To express the ligand binding domain of human TLR9 in an active ligand-binding form.
2. To express sufficient quantities of recombinant TLR9 protein for biochemical characterization.
3. To use recombinant human TLR9 to analyze ligand binding characteristics.

3.0 MATERIALS AND METHODS

3.1 Reagents, Supplies and Equipment

Biological and chemical reagents and supplies used in the experiments throughout this thesis along with their commercial suppliers are listed in **Table 3.1**.

3.2 Plasmid Construction/Cloning

With some species-specific variations, TLR9 is a protein of approximately 1030 amino acids; the N-terminal 750 residues contain 25 LRR motifs, residues 750-810 an extracellular C-terminal motif, residues 810-825 represent the predicted transmembrane region and residues 825-1030 the intracellular TIR domain. As the ligand-binding region of TLR9 has not been well-characterized, we selected a region encompassing the entire ectodomain, amino acids 1-815, to represent the ligand-binding domain (LBD). Although amino acids 810-815 are predicted to lie within the transmembrane domain, we did not anticipate problems with the recombinant protein, such as solubility.

DNA encoding the ectodomain was PCR amplified, with the addition of a six residue histidine-tag at the carboxy terminus (see **Table 4.1**, page 80), using a plasmid containing the human TLR9 open-reading frame as a template (kindly provided by Dr. Grayson Lipford, Coley Pharmaceuticals, Wellesley, MA). The resulting PCR product was cloned downstream of a bovine hsp70A promoter and the expression cassette stably integrated into Madin-Darby bovine kidney epithelial (MDBK) cells by a lentiviral delivery system (Rouas, Uch et al. 2002).

Table 3.1. List of selected chemical supplies, enzymes, and proteins.

Chemicals	Supplier
Acetic Acid	EMD
Acrylamide	Sigma
Agarose, electrophoresis grade	Invitrogen
Ammonium chloride	J.T. Baker
Ammonium persulfate (APS)	J.T. Baker
Antibiotic/antimycotic solution	Gibco
N,N-Bis(2-hydroxyethyl)-glycine	Sigma
Bromophenol Blue	Sigma
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Sigma
Chloroform:isoamyl alcohol	Amersham Biosciences
Coomassie Blue G250	Biorad
Dithiothreitol (DTT)	Biorad
Dulbecco's modified Eagle's medium (DMEM)	Gibco
Ethidium bromide	Pharmacia Biotech
Ethylenediamine tetraacetic acid (EDTA)	Fluka BioChemika
Fetal Bovine Serum (FBS)	Gibco
Glycerol	Amersham Bioscience
Glycine	ICN Biomedicals
Magnesium Chloride	Sigma
2-mercaptoethanol	Sigma
Methanol	EMD
N,N'-Methylene-bis-acrylamide	ICN Biomedicals
p-nitro-blue-tetrazolium (NBT)	Sigma
Phenol (glass distilled)	Sigma

Sodium acetate	Sigma
Sodium chloride	Sigma
Sodium dodecyl sulfate	EMD
Sodium hydroxide	EMD
Sodium phosphate monobasic	EMD
Sodium phosphate dibasic	EMD
N,N,N',N'-Tetramethylethlene-diamine (TEMED)	Pharmacia Biotech
TRIzol Reagent	Invitrogen
Tween 20	Biorad
Enzymes	**Suppliers**
PNGase F	New England Biolabs
Trypsin	Boehringer Ingelheim
Antibodies	**Supplier**
α-TLR9 mouse mAB	EMD
Goat-α-mouse IgG	Kirkegarrd & Perry Laboratories
Plasmid and ODNs	**Supplier**
pFLAG-CMV2	Sigma
PD-ODNs	Invitrogen

Supplier	**Addresses**
Amersham Pharmacia	Amersham Biosciences, Pittsburgh, PA, USA.
Biorad	Bio-Rad Laboratories Ltd., Mississauga, ON, Canada

Boehringer Mannheim	Boehringer Ingelheim Ltd., Laval, QC, Canada.
EMD Bioscience	VWR Canlab, Mississauga, ON, Canada.
Fluka BioChemika	Sigma-Aldrich Canada Ltd., Oakville, ON, Canada.
Gibco	Invitrogen Ltd., Gaithersburg, MD, USA.
ICN Biomedicals	ICN Biomedical Canada Ltd., Saint Laurent, PQ, Canada.
Invitrogen	Invitrogen Canada, Inc., Burlington, ON, Canada.
J.T. Baker	Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA.
Kirkegarrd & Perry Laboratories, Inc.	KPL, Inc., Gaithersburg, MD, USA.
New England Biolabs	NEB Ltd., Pickering, ON, Canada.
Novagen	VWR Canlab, Mississauga, ON, Canada.
Pharmacia Biotech	Pfizer Canada, Inc., Mississauga, ON, Canada.
Sigma	Sigma-Aldrich Canada Ltd., Oakville, ON, Canada.

3.3 Heat Shock System

3.3.1 Vector System and Cell Line Creation

A eukaryotic expression system utilizing the bovine hsp70A gene promoter to direct the heat-regulated synthesis of proteins in transfected bovine kidney epithelial cells was used to express human TLR9(LBD), as described (**Figure 3.1**).

Stably transfected MDBK cells were obtained from Dr. Robert Brownlie (VIDO), who, having used the lentiviral delivery system, performed cloning of our isolated TLR9(LBD). Briefly, the cell line was made by transducing MDBK cells with a pseudolentivirus containing the construct. To make the pseudovirus, human embryonic kidney (HEK) 293T cells were transfected with three plasmids: one containing the VSG envelope protein, one containing packaging functions and the other containing the TLR9(LBD) flanked by HIV long terminal repeat (LTR) regions. The lentiviral vector also contained the gene encoding resistance to neomycin to aid in the selection process.

3.3.2 Cell Line Maintenance

MDBK cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (10,000 U penicillin, 10 mg streptomycin, 25 μg

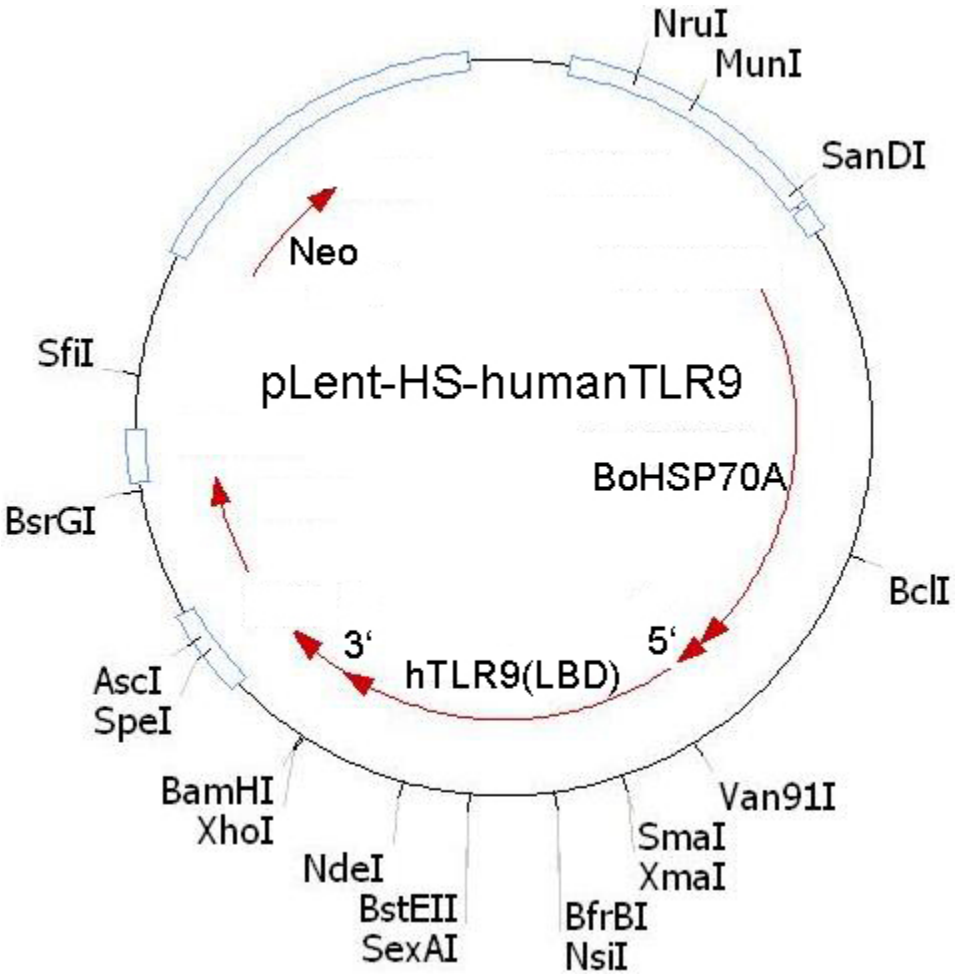

Figure 3.1. Expression of hTLR9(LBD). The expression plasmid pLent-HS-humanTLR9 containing the bovine hsp70A heat-shock gene promoter (BoHSP70A). The promoter was used to direct the heat-regulated synthesis of human TLR9 ligand binding domain [hTLR9(LBD)] in stably transfected bovine kidney (MDBK) cells. A neomycin resistance cassette (Neo) was included in the plasmid for positive selection of transfected cells. Restriction endonuclease cut sites are shown on the external ring.

amphotericin B per 25 mL) in Corning T75 culture flasks and incubated at 37°C/5% CO_2.

Confluent cell monolayers were treated with trypsin, a serine protease used to re-suspend cells adherent to tissue culture dishes. Media was removed from a flask containing a confluent monolayer of cells and cells were rinsed with 5 mL of Versene to remove residual FBS, which may inhibit the action of trypsin, and to increase the effect of trypsin by taking up calcium and magnesium ions. Another 5 mL of Versene plus approximately 1 mL of 5% trypsin was added to the flask and the cap tightened. After 30 second incubation the Versene/trypsin solution was removed from the flask, the cap tightened slightly and the flask incubated at 37°C in a 5% CO_2 atmosphere for 1-2 minutes, or until cell detachment was observed. Ten milliliters of fresh DMEM supplemented with FBS and antibiotic/antimycotic solution (as above) was added to the flask to resuspend the cells.

On day 0, cells were grown to confluence in a single T75 culture flask. The next day (day 1), cells were split in to two T150 culture flasks. After incubating for two days to allow cells to become confluent (day 3), cells were split 1:2 in to four T150 flasks. Two days following (day 5), cells were split 1:4 in to sixteen T150 flasks. Three days later (day 8), cells were split 1:2 in to 32 T150 flasks and the next day (day 9) cycle one of heat-shock began. Ten cycles of heat shock followed (days 10 through 18).

3.4 Overexpression of TLR9(LBD)

Confluent cells in thirty-two T150 culture flasks were washed twice in serum-free medium to remove serum from the cells, and incubated at 39°C/5%CO_2 to induce

protein expression. At the end of a 6 hour incubation period, flasks were returned to $37^{o}C/5\%CO_2$ for 18 hours to allow expression of the protein. At the end of a 24 hour cycle, the media was collected and centrifuged for 5 minutes at 2,000xg to remove cells and debris. Culture medium was replaced with fresh serum-free DMEM and the cycle repeated 10 times. Every 24 hour cycle yielded 640-800 mL of media (approximately 0.1 mg of protein per liter as determined by Bradford protein quantification).

An advantage of this expression system is that the recombinant protein is secreted into the culture medium, greatly facilitating collection and further purification. Although yields of the protein are relatively low, the non-destructive nature of this system permits large-scale production by splitting the culture and though ongoing heat shock cycling. Relatively large quantities of the protein could be produced from ten rounds of temperature shifting with thirty-two T150 culture flasks.

The secreted TLR9(LBD), containing a histidine-tag, was then purified using commercially available Nickel-columns (His-Bind Quick 300, Novagen). Buffer compositions were as follows: 8x binding buffer, pH 7.9 [23.38% (w/v) NaCl, 16% (v/v) Tris-HCl, 0.27% (w/v) imidazole]; 8x wash buffer, pH 7.9 [23.38% (w/v) NaCl, 16% (v/v) Tris-HCl, 3.27% (w/v) imidazole]; 4x elution buffer, pH 7.9 [11.69% (w/v) NaCl, 8% (v/v) Tris-HCl, 27.22% (w/v) imidazole]. Seven milliliters 1x binding buffer, 2.5 mL 1x wash buffer and 1 mL 1x elution buffer were prepared (per cartridge) by diluting stocks to 1x with ddH_2O. One His-Bind cartridge was attached to a 10cc syringe and buffers pushed through at a rate of approximately 2 drops per second. The cartridge was first wet and equilibrated with 2 mL 1x binding buffer. Media containing secreted TLR9(LBD) was passed through the column 5 mL at a time (total volume: 20

mL) and then the cartridge washed with 5 mL 1x binding buffer. The cartridge was washed with 2.5 mL 1x wash buffer prior to elution with 1 mL 1x elution buffer.

Following this purification a single protein species was observed, which was bound by a monoclonal antibody directed against the extracellular domain of human TLR9, demonstrated by Western blotting. However, our assays were not dependent on the absolute purity of the protein, and as such were carried out with protein which had not been purified with a Nickel-column.

3.5 Western Blot Analysis

3.5.1 SDS-PAGE

Protein samples were denatured and resolved through a 10% SDS gel (10% bis-acrylamide, 25% 1.5 M Tris pH 8.8 (v/v), 0.1% SDS, 48.4% (v/v) ddH_2O, 0.05% APS, 0.01% TEMED) with a 4% stacking gel [4% bis-acrylamide, 25% (v/v) 0.5 M Tris-HCl pH 6.8, 0.1% SDS, 63.4% (v/v) ddH_2O, 0.05 % APS, 0.01% TEMED] in 1x SDS running buffer [0.3% (w/v) Tris base, 1.44% (w/v) glycine, 0.1% (w/v) SDS]. Samples were run at 90 V through the stacking gel and 180V through the resolving gel.

The SDS gel was stained using a Coomassie G250 staining protocol. The gel was incubated in fixation solution [50% (v/v) ethanol, 2% (v/v) orthophosphoric acid, 48% (v/v) ddH_2O] for 2.0 hours and subsequently incubated in G250 stain solution [34% (v/v) methanol, 17% (w/v) ammonium sulfate, 2% (v/v) orthophosphoric acid, 0.1% (w/v) Coomassie G250, 64% (v/v) ddH_2O] for a minimum of 4 hours, up to 16-18 hours. Following staining the gel was rinsed 1x with destaining solution [25% (v/v)

methanol] and destained for 1-2 hours, with an absorbing sponge placed in the destain for more complete destaining.

A gel was run in parallel without staining for use in Western Blots.

3.5.2 Western Blots

After resolution through the SDS gel, proteins were electrophoretically transferred to a nitrocellulose membrane essentially as described (Towbin, Staehelin et al. 1979). Nonspecific binding sites were blocked by incubating the nitrocellulose for 1.0 hour in TBST buffer (0.1 M Tris-HCl, 170 mM NaCl, 0.05% Tween-20 pH 7.5) containing 3% skim milk. Membranes were incubated for 1 hour in primary antibody (α-human TLR9 diluted to 2 μg/mL in TBST) and then for 1 hour with alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (0.5 μL/mL in TBST). After being washed, membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate nitro-blue tetrazolium substrate (BCIP/NBT). Anti-human TLR9 was obtained from Oncogene Research Products (EMD Biosciences) and goat anti-mouse IgG from Kirkegarrd and Perry Laboratories, Inc.

3.5.3 Deglycosylation of TLR9(LBD)

Purified TLR9(LBD) was treated with peptide N-glycosidase F (PNGase F) as follows. Twenty μg of TLR9(LBD) was denatured in 5% SDS with 0.4 M dithiothreitol (DTT) for 10 minutes at 100^{o}C. Following this, reaction buffer (0.5 M sodium phosphate, pH 7.5) and 10% NP-40 were added at 1/10 reaction volume with 2.5 μL of PNGase F (0.278 mg/mL). The deglycosylation was allowed to proceed at 37^{o}C for 1

hour. Samples were visualized by SDS-PAGE and Western blotting, as described previously (sections 3.5.1 and 3.5.2).

3.6 Agarose Electrophoretic Mobility Shift Assays

3.6.1 DNA Binding Assays

Unless otherwise specified, DNA binding assays were performed with the incubation of 8 μg TLR9(LBD) (20 mM PBS pH 7.2, 100 mM NaCl, 0.5 mM β-mercaptoethanol) with 1.4 μg DNA [pFLAG-CMV2 expression vector (Sigma) (**Figure 3.2**)] in the presence of 10 mM $MgCl_2$. Reactions were buffered with 100mM sodium acetate (pH 5.2) to a final volume of 50 μL. Reactions were incubated at 37^{o}C for 10 minutes and stopped with the addition of 10 μL 30% glycerol and immediately electrophoresed.

3.6.2 Agarose Gels

DNA binding assays were electrophoresed through a 0.8% agarose gel in TAE buffer [24.2% (w/v) Tris, 5.7% (v/v) glacial acetic acid, 50 mM EDTA] at 95 V for 1-2 hours. DNA was visualized by ethidium bromide staining: 1-3 μL of ethidium bromide was added to each 75 mL agarose gel prior to electrophoresis.

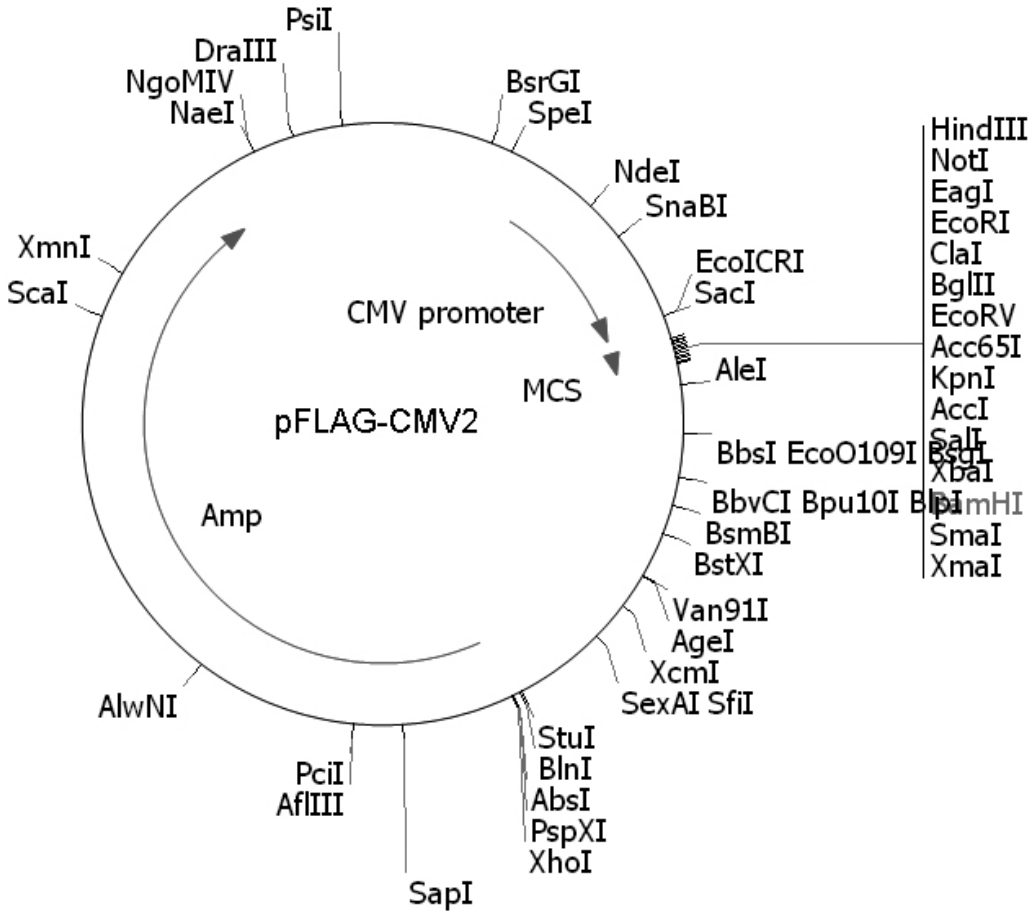

Figure 3.2. pFLAG-CMV2 used in binding assays. Expression is driven by the CMV promoter, and restriction endonuclease cut sites are shown on the external ring. The multiple cloning site (MCS) and ampicillin resistance cassette (Amp) are shown. The plasmid contains six CpG motifs to serve as a ligand for human TLR9.

3.6.3 Supershift Assays

Supershift assays were performed by the same methodology described for the mobility shift assays with the exceptions that the TLR9(LBD) was dialyzed against 50 mM PBS pH 7.2, 100 mM NaCl and reactions were performed at pH 7.2 (50 mM Tris) to remove any small molecules which may influence the strength and specificity of the DNA:protein interaction. The TLR9 monoclonal antibody used was the same as that used in Western blot analysis. The reaction mixture was incubated for 1 hour at 37^{o}C prior to electrophoresis.

3.6.4 Freeze-Squeeze Method for Protein Extraction from Agarose

Following agarose electrophoresis, bands corresponding to the nucleoprotein complexes were excised and placed in microcentrifuge tubes. Tubes were frozen at -80^{o}C overnight. Samples were thawed at room temperature and centrifuged at 14,000 rpm for 10 minutes. The supernatant was extracted for further analysis.

3.6.5 Oligodeoxynucleotides

The sequences of ODNs used in this investigation are presented in **Table 3.2**. PD-ODNs were purchased from Invitrogen.

3.7 Establishment of Toll-like Receptor 9 Knockout Cell Line

3.7.1 Generation of Knockout Mice

TLR9$^{-/-}$ mice were generated by Hemmi *et al* (Hemmi, Takeuchi et al. 2000) by disrupting the TLR9 gene with homologous recombination, and bred on a C57Bl/6

Table 3.2. Oligodeoxynucleotides used in this investigation.

ODN Name	Sequence
Human CpG	GGATCAGCGGAGGTCGTTTTGTCGTTCTCTGTC
Human GpC	GGATCGACAGAGAACGACAAAACGACCTCCGCT
Mouse CpG	GGATCAGCGGAGGACGTTCTGACGTTCTCTGTC
Mouse GpC	GGATCGACAGAGAACGTCAGAACGTCCTCCGCT

background. Breeding pairs of TLR9$^{-/-}$ mice were supplied to VIDO by Dr. Heather Davis and mice were bred at VIDO.

3.7.2 Generation of Cell Line

The fibroblast cell line was generated from cells which grew out of tissue collected from the abdominal wall (muscle and subcutaneous tissue) of TLR9$^{-/-}$ mice. Cells were initially maintained in Minimum Essential Media (MEM) supplemented with 10% FBS and 1% antibiotic/antimycotic solution (10,000 U penicillin, 10 mg streptomycin, 25 μg amphotericin B per 25 mL). After one week, cells were maintained in MEM + 20% FBS + 1% antibiotic/antimycotic solution in order to enrich the culture, as cell proliferation, in most cases, increases with increasing concentrations of FBS. Cells were passaged until fibroblasts became the dominant cell type in culture, at approximately seven weeks.

3.7.3 Confirmation of TLR9$^{-/-}$ Cells

3.7.3.1 Protein Isolation

TLR9$^{-/-}$ cells were seeded at 2.5 x 10^4 cells per well in 24-well plates and incubated at 37^{o}C/5%CO_2 for two days, until confluent. Following 5 minute centrifugation at 2,500 rpm, to pellet the cells, and rinsing with 0.5 mL PBS, cells were lysed directly in culture plates by adding 500 μL TRIzol reagent (Invitrogen) to each well, and passing the cell lysate through a pipette. Lysed samples were incubated for 5 minutes on ice and then 5 minutes at room temperature (15^{o}C to 30^{o}C, according to manufacturers protocol) to permit complete dissociation of nucleoprotein complexes.

The lysates from two replicate wells were pooled into an Eppendorf tube and 200 μL of chloroform was added per 1.0 mL of TRIzol reagent (200 μL chloroform per tube). Tubes were shaken vigorously for approximately 15 seconds and incubated 2-3 minutes at room temperature. Samples were then centrifuged at 12,000xg for 15 minutes at 2-8^{o}C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The colorless (RNA) phase and interphase (DNA) were removed, and 300 μL 100% ethanol added to the red phenol-chloroform portion of each tube in order to precipitate any remaining DNA. Samples were inverted to mix, and incubated 2-3 minutes at room temperature. Following 5 minute centrifugation at 2,000xg the supernatant was collected for protein isolation. Proteins were precipitated from the phenol-ethanol supernatant with 1.5 mL isopropyl alcohol added per tube. Samples were stored for 10 minutes at room temperature, and the protein precipitate sedimented at 12,000xg for 10 minutes at 2-8^{o}C. The supernatant was removed and the pellet in each tube washed 3x in a 1.5 mL of a solution containing 0.3 M guanidine hydrochloride in 95% ethanol. During each wash cycle, samples were incubated for 20 minutes at room temperature then centrifuged at 7,500xg for 5 minutes at 2-8^{o}C. After the final wash, protein pellets were vortexed in 1.5 mL ethanol and incubated at room temperature for 20 minutes. Following this incubation, samples were centrifuged at 7,500xg for 5 minutes at 2-8^{o}C. Pellets were air dried or vacuum dried then dissolved, by adding 100 μL 1% SDS per tube. Complete dissolution of the protein pellet in some cases required incubating the sample at 50^{o}C. Samples were centrifuged at 10,000xg for 10 minutes and the supernatants transferred to fresh Eppendorf tubes for storage at -5^{o}C to -20^{o}C.

Western blots were carried out as described previously.

3.7.4 Transfections

Fibroblast cells were transfected with a GFP-expressing plasmid using Fibroblast Transfection Reagent (Altogen Biosystems) in 24-well plates. Two to five microliters of transfection reagent was incubated with 500 ng-1 μg pCANGFP DNA in 40 μL serum-free MEM for 15-30 minutes. Cells were plated at $2.5x10^4$ cells/well (in MEM + 10% FBS) and transfection complexes were added (after incubation). Plates were incubated at $37^oC/5\%CO_2$ for 24 hours, after which the media was replaced with fresh MEM + 10% FBS. Transfection efficiency was assessed at 48h post-transfection and 72h post-transfection by staining the nuclei with 4'-6-Diamidino-2-phenylindole (DAPI) and visualizing using a fluorescence microscope.

4.0 RESULTS

4.1 Expression and Purification of Toll-like Receptor 9

In spite of the considerable therapeutic interest in TLR9 it has been difficult to design and screen TLR agonists due to the technical difficulties associated with purification of the receptor for biochemical characterization, including the production of the protein in its active form, with conservation of the normal post-translational modifications found on the endogenous receptor. Accordingly, little information is available regarding the structural and ligand-binding properties of TLR9 and most of the assumptions regarding TLR9 ligand binding specificity have been extrapolated from cell stimulation experiments.

Here, the ectodomain of human TLR9 [TLR9(LBD)] was expressed as a soluble protein by using a novel expression system for the purpose of investigation of its ligand-binding properties. The primary objective in the expression of the TLR9(LBD) was to obtain sufficient quantities of the active protein for biochemical characterization. As the assay system that we employed to characterize nucleic acid binding by TLR9 is not dependent upon absolute purification of the receptor, this enabled us to focus on the generation of sufficient quantities of the active ligand-binding domain. The caveat of these assays is that they require large amounts of protein. We investigated the use of a proprietary eukaryotic heat shock system established at VIDO which has proven successful for the expression of other glycosylated proteins (Kowalski, Gilbert et al. 1993).

4.1.1 Over-expression via Heat Shock

The expression of human TLR9 in mammalian cells using this heat shock system was the proposed solution to the problems associated with purification of TLRs, as it has certain advantages: a) bacterial expression systems do not have the cellular machinery in place to glycosylate proteins; using a eukaryotic expression system, the protein is produced in a glycosylated form, b) the protein is secreted into media and is, therefore, easy to collect and c) the system is robust in that, although there is low protein yield with each cycle of heat shock, the cycle can be repeated upwards of fifty times and d) the secretion of the protein into the media provides an efficient means to separate the recombinant protein from cellular proteins.

The heat-shock vector system is highly inducible. Initial experiments at VIDO using this heat shock system to direct synthesis of proteins were carried out at 43^{o}C, although lower induction temperatures had been shown to be more effective for certain proteins. Our experiments were carried out with inductions at both 42^{o}C as well as 39^{o}C. Results from our initial experiments indicated that the induction of heat shock at 39^{o}C was preferable to induction at 42^{o}C, as synthesis of larger amounts of protein was visible on the SDS gels (**Figure 4.1**). Cells were maintained at 37^{o}C, as is the optimal growth temperature for this cell type. Of note, the protein visible in the SDS gels is bovine serum albumin (as confirmed by mass spectrometry, data not shown). The amount of TLR9(LBD) protein produced is very small and is therefore difficult to visualize on an SDS gel.

Madin-Darby bovine kidney (MDBK) cells were initially transduced with a pseudolentivirus containing our TLR9(LBD). The final lentiviral vector contained the

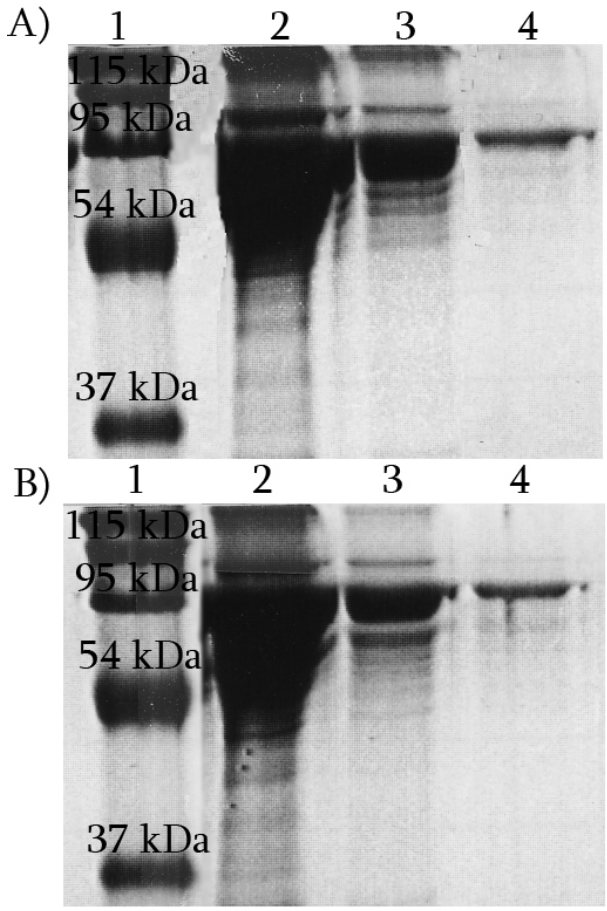

Figure 4.1. A) SDS-PAGE of media collected from heat shock inductions at 42°C. Proteins were resolved through a 4% stacking gel followed by a 12% running gel. Lane 1 - broad range protein biomarker. Lane 2 – 250 μg protein from supernatant. Lane 3 – 50 μg protein from supernatant. Lane 4 – 10 μg protein from supernatant. **B) SDS-PAGE of media collected from heat shock inductions at 39°C.** Proteins were resolved through a 4% stacking gel followed by a 12% running gel. Lane 1 - broad range protein biomarker. Lane 2 – 250 μg protein from supernatant. Lane 3 – 50 μg protein from supernatant. Lane 4 – 10 μg protein from supernatant. Proteins were visualized via staining with Coommassie blue G250 stain as described (Section 3.5.1).

gene encoding resistance to neomycin to aid in the selection process. Cells which survived treatment with neomycin were propagated and maintained as described.

For our purposes, thirty-two T150 culture flasks, each containing 20-23 mL of media, were used for each cycle of heat-shock. The cycle was repeated ten times (**Figure 4.2**), generating approximately 7L of media.

4.2 Western Blot Analysis

The presence of a soluble form of the receptor in the media was confirmed through Western blot analysis. The primary antibody was a monoclonal antibody corresponding to amino acid residues 268-284 of human TLR9. Through Western blot analyses we have shown that the antibody binds our recombinant human TLR9(LBD) (**Figure 4.3**), whereas media collected from untransfected control cells was unreactive towards the antibody. It is therefore unlikely that the monoclonal antibody was reacting with contaminating cellular proteins. We observed a single protein species of approximately 170 kDa, which is considerably larger than that calculated for the protein component of the recombinant receptor of approximately 91 kDa (**Table 4.1**). However, TLR9 is known to be highly glycosylated, and the single species observed has a mass consistent with previous reports for glycosylated TLR9 (Latz, Schoenemeyer et al. 2004).

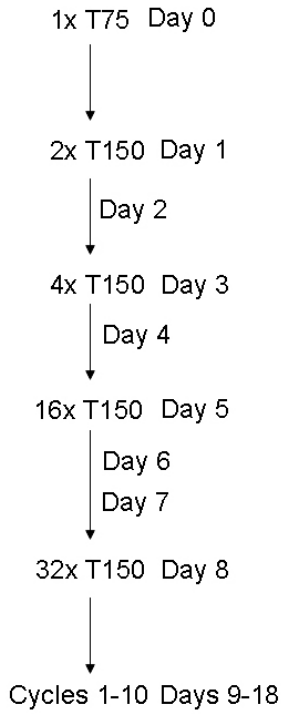

Figure 4.2. Schedule and induction of hTLR9(LBD). Day 0: cells are grown to confluence in a single T75 culture flask. Day 1: cells are split in to two T150 culture flasks. Day 3: cells are split 1:2 in to four T150 flasks. Day 5: cells are split 1:4 in to sixteen T150 flasks. Day 8: cells are split 1:2 in to 32 T150 flasks. Days 9-18: cycles 1 through 10 of heat shock.

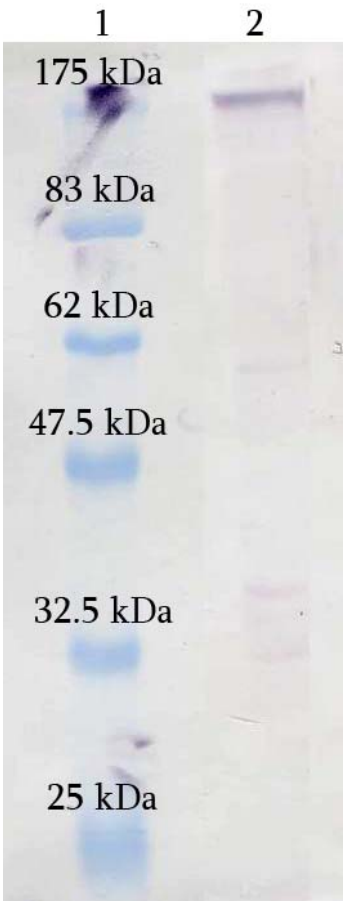

Figure 4.3. Western blot analysis of hTLR9(LBD). Medium was collected from heat shocked cells and proteins were resolved through a 10% SDS-polyacrylamide gel and analyzed via Western blot with a monoclonal antibody to human TLR9. Lane 1 – broad range protein biomarker. Lane 2 – 10 μg protein from supernatant.

Table 4.1. Amino acid sequence of the hTLR9(LBD). Calculated molecular weight: 90.55 kDa with predicted glycosylation sites in boldface type and underlined (Weber, Morse et al. 2004), and histidine tag (6xH) at the carboxy terminus.

1 MGFCRSALHP LSLLVQAIML AMTLALGTLP AFLPCELQPH GLVNCNWLFL KSVPHFSMAA

61 PRG**N**VTSLSL SSNRIHHLHD SDFAHLPSLR HLNLKWNCPP VGLSPMHFPC HMTIEPSTFL

121 AVPTLEEL**N**L SYNNIMTVPA LPKSLISLSL SHTNILMLDS ASLAGLHALR FLFMDGNCYY

181 KNPCRQALEV APGALLGLG**N** LTHLSLKYN**N** LTVVPRNLPS SLEYLLLSYN RIVKLAPEDL

241 A**N**LTALRVLD VGGNCRRCDH APNPCMECPR HFPQLHPDTF SHLSRLEGLV LKDSSLSWL**N**

301 ASWFRGLGNL RVLDLSENFL YKCITKTKAF QGLTQLRKL**N** LSFNYQKRVS FAHLSLAPSF

361 GSLVALKELD MHGIFFRSLD ETTLRPLARL PMLQTLRLQM NFINQAQLGI FRAFPGLRYV

421 DLSDNRISGA SELTATMGEA DGGEKVWLQP GDLAPAPVDT PSSEDFRP**N**C STL**N**FTLDLS

481 RNNLVTVQPE MFAQLSHLQC LRLSHNCISQ AV**N**GSQFLPL TGLQVLDLSH NKLDLYHEHS

541 FTELPRLEAL DLSYNSQPFG MQGVGH**N**FSF VAHLRTLRHL SLAHNNIHSQ VSQQLCSTSL

601 RALDFSGNAL GHMWAEGDLY LHFFQGLSGL IWLDLSQNRL HTLLPQTLRN LPKSLQVLRL

661 RDNYLAFFKW WSLHFLPKLE VLDLAGNQLK ALT**N**GSLPAG TRLRRLDVSC NSISFVAPGF

721 FSKAKELREL **N**LSANALKTV DHSWFGPLAS ALQILDVSAN PLHCACGAAF MDFLLEVQAA

781 VPGLPSRVKC GSPGQLQGLS IFAQDLRLCL DEALSHHHHHH

4.2.1 Glycosylation

Glycosylation is important for the function of TLRs and that it plays a role in influencing receptor surface presentation, trafficking and pattern recognition (Weber, Morse et al. 2004). All of the TLRs are predicted to be glycosylated and as would be expected, these glycosylation sites are said to be present within the extracellular domains of the receptors. For example, it has been demonstrated that glycosylation is important for the function of TLR3, as modifying glycosyl groups has a negative effect on TLR3 activity (Jingchuan, Duffy et al. 2006). The convex (outer) surface of the ectodomain of TLR3, however, is proposed to be completely devoid of glycosylation, and therefore is predicted to be the site of ligand-binding.

Weber predicted thirteen potential glycosylation sites for TLR9 (Weber, Morse et al. 2004) (**Table 4.1**). As with other TLRs, these sites are presumably important for the function of the receptor, which is why the production of a glycosylated protein was important here.

Glycosylation of the recombinant protein was verified using PNGase F (N-glycosidase F) to remove *N*-linked sugar residues. This deglycosylation resulted in a molecular weight shift to between the 83 kDa and the 175 kDa band (**Figure 4.4**). Though it is difficult to extrapolate a molecular weight between 83 and 175 kDa, the apparent molecular weight of the deglycosylated receptor is in agreement with the calculated weight of the recombinant protein (91 kDa), taking in to account that the protein may not have been completely deglycosylated.

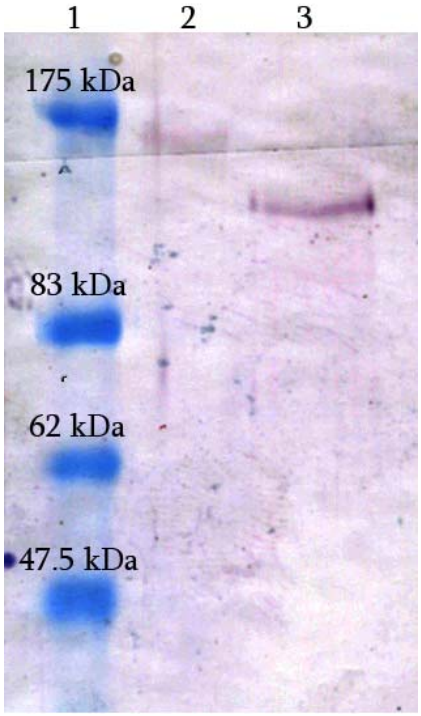

Figure 4.4. Western blot analysis of glycosylated and deglycosylated hTLR9(LBD). Proteins were resolved through a 7% SDS-polyacrylamide gel. Lane 1 – broad range protein biomarker. Lane 2 – 10 μg protein from supernatant. Lane 3 – 20 μg protein from supernatant, treated with an N-glycosidase (PNGase F). There is an obvious difference in molecular weight between the deglycosylated and glycosylated samples.

4.3 Agarose Electrophoretic Mobility Shift Assays

Previous investigations of the ligand binding properties of TLR9 have relied on SPR technology. One drawback to this approach is that either the receptor or the ligand is required to be immobilized on to a solid surface, which does not allow the complex receptor-ligand or receptor-receptor interactions to take place as they would *in vivo*. A system which did not require immobilization of either receptor or ligand was designed in order to maintain the dynamic relationship between receptor and ligand. Within agarose gels, plasmids normally resolve to two predominant species: a faster-moving supercoiled form and a slower-moving relaxed form (Oppenheim 1981).

Through the use of a modified agarose electrophoretic moblity shift assays, we have been able to show that TLR9(LBD) binds plasmid DNA. This assay provides information regarding nucleoprotein complex formation based on altered patterns of DNA migration in the presence and absence of a DNA binding protein. In contrast to the acrylamide shift assays, this system allows large nucleoprotein complexes to be resolved. It also allows high-throughput analysis in a manner that is not dependent on immobilization of the receptor or the ligand. This permits more characterization of receptor/ligand dynamics with respect to the formation of higher order structures (Ozer, Mitsouras et al. 1998). Within this system, patterns of nucleoprotein migration are dependent upon the charge to mass ratio of the complexes, as well as the formation of higher order structures that influence the overall size of the molecule. Therefore, there is not a linear relationship between the calculated molecular mass and the distance migrated in the gel. The shifts are quantitative in nature, permitting discrimination and visualization of nucleoprotein complexes of differing compositions. This is of particular

value for discrimination of nucleic acid binding by monomeric or dimeric forms of proteins as these complexes migrate as distinct entities.

The TLR9(LBD) influences the migration of plasmid DNA, as shown by the formation of two distinct migrating species (**Figure 4.5A**). It is of note that the faster moving unbound supercoiled plasmid is decreased in concentration. It is likely that these two nucleoprotein species represent different stoichiometries of TLR9 binding to the plasmid. These novel species are hypothesized to represent monomeric and dimeric forms of the receptor bound to a single plasmid. These two species have since been verified as the monomeric and dimeric forms of the receptor binding the plasmid. The concentrations of plasmid DNA in both the proposed monomeric and dimeric species were confirmed to be equivalent by quantification of band intensity using the AlphaEase imaging system. Quantification is based on band intensity using ethidium bromide staining. Ethidium bromide is an intercalating agent which fluoresces under ultraviolet light, and binds uniformly to linear DNA molecules. In our assay, we quantified the supercoiled form of the plasmid. It has been demonstrated that ethidium bromide causes unwinding of DNA, without cutting (Radloff, Bauer et al. 1967), so we did not anticipate that there would be significant differences in band intensities due differences in the amount of ethidium bromide bound. To determine the relative concentration of TLR9(LBD) present within each complex, the shifted bands were excised and proteins extracted by the freeze-and-squeeze method. Western blot analysis with the monoclonal human TLR9 antibody confirmed that the intensity of the signal in the lane corresponding to the proposed dimeric form of the receptor is 1.95 times the inensity of the signal in the lane corresponding to the monomeric form of TLR9 (**Figure 4.5B**).

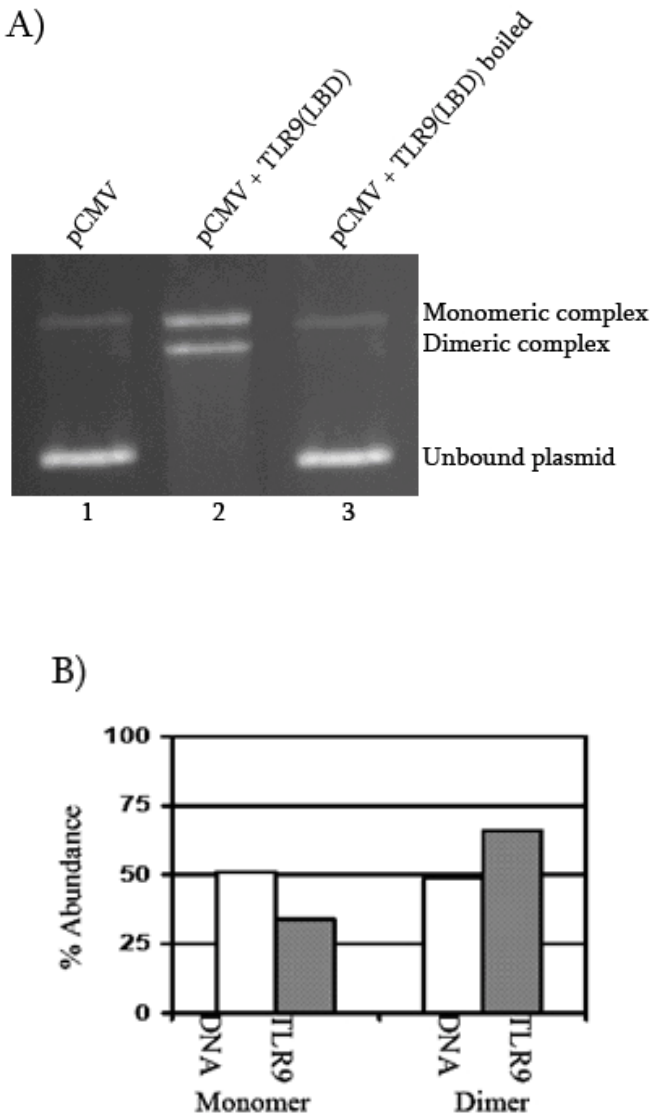

Figure 4.5. A) Plasmid binding by hTLR9(LBD). pCMV and TLR9(LBD) are used in constant quantities of 1.4 μg and 8 μg, respectively, in the presence of 10 mM $MgCl_2$. Reactions were buffered with 100 mM sodium acetate (pH 5.2) and incubated at 37°C for 10 minutes and stopped with the addition of 30% glycerol. Lane 1: pCMV; Lane 2: pCMV and TLR9(LBD), and Lane 3: pCMV and TLR9(LBD) boiled for 15 minutes prior to addition. **B) Confirmation of monomeric and dimeric nucleoprotein complexes.** The stoichiometry of the shifted nucleoprotein complexes was determined by comparing relative abundances of both nucleic acid and TLR9(LBD) within uniquely migrating species hypothesized to represent monomeric and dimeric forms of the TLR9 nucleoprotein complex. Relative band intensities were calculated for both the ethidium stained agarose gel to determine relative nucleic acid content as well as for Western blots of proteins extracted from these gel slices. Results represent the averages of two trials.

This dimerization is not surprising as it has been shown that ligand-induced dimerization is a common theme within the Toll-like family (Choe, Kelker et al. 2005; Gay, Gangloff et al. 2006).

Maintenance of a correctly folded structure is also important in plasmid binding. Deliberate disruption of the structure of the TLR9(LBD), by boiling for example, prior to its addition to the reaction mixture abolishes DNA binding activity (**Figure 4.5**, Lane 3). This demonstrates that the recombinant TLR9(LBD) is functional in terms of ligandbinding and can bind double-stranded DNA, a point of contention in the literature. The ligand used in this assay system is a closed circular plasmid which is present in both relaxed and supercoiled forms. Importantly, the TLR9(LBD) was observed to associate preferentially with the supercoiled forms of plasmids. This may account for the discrepancies reported with respect to the efficiency of plasmid binding by TLR9. Investigations reporting minimal association with plasmid have employed linear, double-stranded ODNs, while those supporting plasmid binding have used circular plasmids (Cornélie, Hoebke et al. 2004; Latz, Schoenemeyer et al. 2004; Rutz, Metzger et al. 2004). The inability of the linear, double-stranded molecules to form supercoiled species would significantly reduce their efficiency as TLR9 ligands. The ability to discriminate the binding of two configurations of the same plasmid is a unique and unexpected advantage of this screening system.

4.3.1 Supershift Assays

To ensure that the differential patterns of migration occurred as a result of the binding of TLR9(LBD), a supershift assay was performed utilizing a monoclonal

antibody to the ectodomain domain of human TLR9 (Kindrachuk, Potter et al. 2007). The incorporation of the monoclonal antibody into the nucleoprotein complex will result in a shifted pattern of migration compared to that of the nucleoprotein complex in the absence of antibody. In the presence of the antibody, a differentially migrating band of the plasmid was observed, confirming the presence of TLR9(LBD) in the nucleoprotein complex (**Figure 4.6**). The ability of the antibody to bind the recombinant protein was verified through conventional Western blot analysis (**Figure 4.3**). The existence of monomeric and dimeric forms of the receptor was confirmed via quantification of band intensity and Western blotting as described (**Figure 4.5B**).

4.3.1.1 ODNs Exert a Cooperative Effect on Plasmid Binding

Using the agarose shift assays, we have also demonstrated that ODNs promote plasmid binding by TLR9. In the absence of ODN, there is no formation of dimeric species observed. This suggests a positive cooperative effect whereby the binding of substrate, in this case nucleic acids, to the binding site causes activation, rather than inhibition, of complex formation. Unlike plasmid DNA, the limited net charge of an ODN is insufficient to cause migration of TLR9(LBD) into an agarose gel. As such, direct visualization of ODN binding is not possible through the agarose gel shift system. It is possible, however, to monitor the association of ODNs with TLR9(LBD) through their impact on TLR9(LBD)-plasmid complexes. Based on the expectation that plasmids and ODNs bind to a conserved site on TLR9, it was anticipated that these molecules would function as competitive inhibitors of each other such that binding of either molecule would prevent binding of the other. Rather than competing with

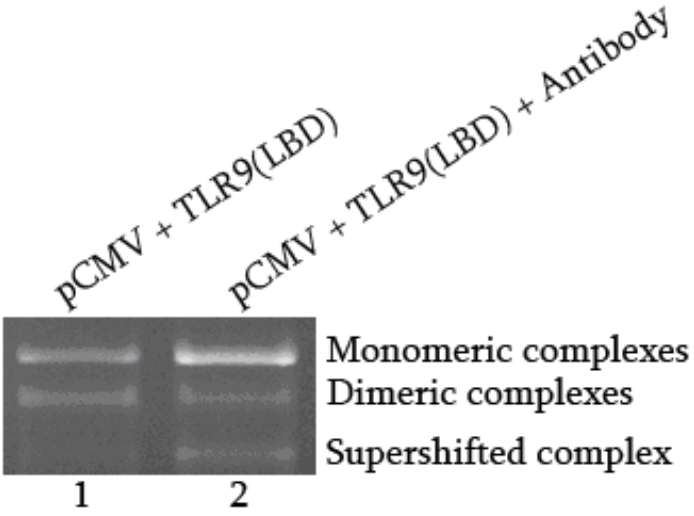

Figure 4.6. Supershift binding assays with anti-TLR9 monoclonal antibody.

Lane 1: pCMV and TLR9(LBD), Lane 2: pCMV, TLR9(LBD) and anti-TLR9 monoclonal antibody (1.5μg). pCMV and TLR9(LBD) are at constant concentrations of 1.4 μg and 8 μg, respectively. Supershift assays were performed by the same methodology described for the agarose shift assays with the exceptions that the TLR9(LBD) was dialyzed against 50 mM PBS pH 7.2, 100 mM NaCl and reactions performed at pH 7.2. The reaction mixture was incubated for 1 hour at 37^{o}C prior to electrophoresis.

plasmid for TLR9(LBD) binding, ODNs were found to promote plasmid binding by TLR9(LBD) (**Figure 4.7**). This effect is independent of sequence as both activating and non-activating ODNs are equally efficient in promoting these effects. This is demonstrative of positive cooperativity as we see the increased formation of dimeric species by titrating in increasing concentrations of ODN while the amounts of plasmid and protein remain the same. The cooperative effect observed with ODNs promoting plasmid binding, as demonstrated with agarose shift assays, also occurs with plasmids promoting the formation of complexes between TLR9(LBD) and ODNs (Kindrachuk, Potter et al. 2007). This supports the conclusion of sensitizing the receptor for ligand binding. Therefore, it can be concluded that nucleic acids, independent of structure or sequence, promote TLR9 dimer formation with either single- or double-stranded nucleic acids able to perform this function.

4.3.1.2 Positive Cooperativity

The mutual cooperativity exerted by nucleic acids, either ODNs or plasmids, on binding of nucleic acids by TLR9(LBD) prompted the consideration of the physiological significance of this phenomenon. Previous investigations of TLR9 activation have utilized cell proliferation assays, providing a valuable platform to monitor the significance of this cooperative effect *in vivo* (Bauer, Heeg et al. 1999; Krieg, Yi et al. 1999). Through cell proliferation assays it was demonstrated that the cooperative effect of nucleic acids on TLR ligand-binding is preserved *in vivo*. The magnitude of cellular responses upon co-stimulation with plasmid and either CpG- or GpC- ODNs was higher than the sum of the individual responses to ODN or plasmid alone

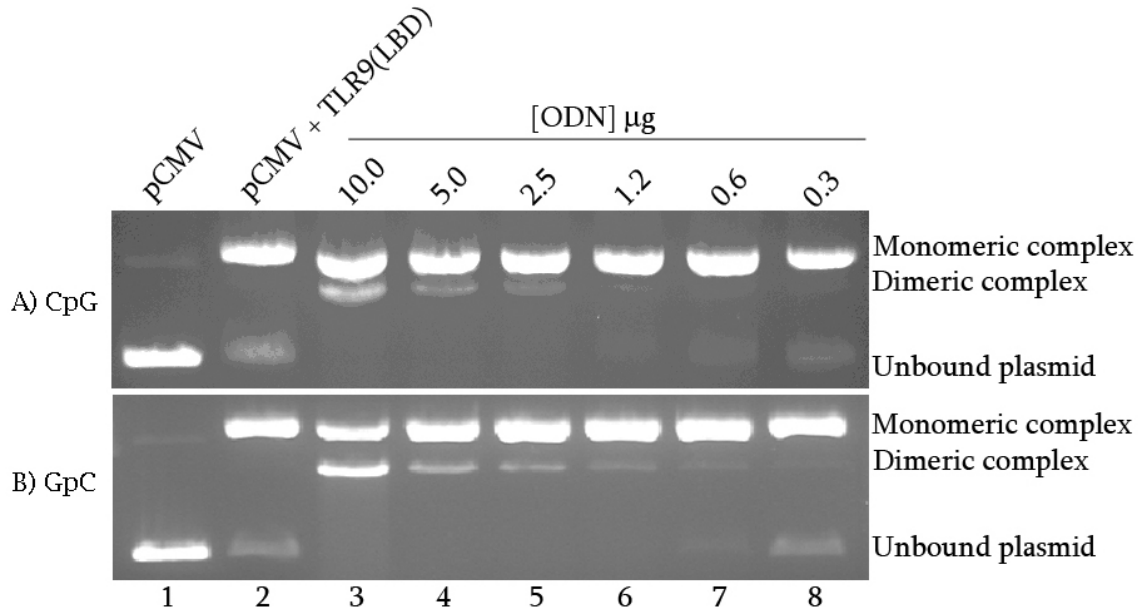

Figure 4.7. A) Cooperative influence of CpG ODNs on plasmid binding. ODNs exert a sequence-independent cooperative effect on plasmid binding. pCMV vector and TLR9(LBD) are used in constant quantities of 1.4 μg and 8 μg respectively. Lane 1: pCMV; Lane 2: pCMV and TLR9(LBD); Lanes 3-8: identical to lane 2 with the addition of serial 2-fold dilutions of CpG PD-ODN starting at 10 μg. **B) Cooperative influence of GpC ODNs on plasmid binding.** ODNs exert a sequence-independent cooperative effect on plasmid binding. pCMV and TLR9(LBD) are used in constant quantities of 1.4 μg and 8 μg respectively. Lane 1: pCMV; Lane 2: pCMV and TLR9(LBD); Lanes 3-8: identical to lane 2 with the addition of serial 2-fold dilutions of GpC PD-ODN starting at 10 μg. All assays were carried out in the presence of 10 mM $MgCl_2$ and buffered with 100 mM sodium acetate. Reactions were incubated at 37^{o}C for 10 minutes and stopped with the addition of 30% glycerol.

(Kindrachuk, Potter et al. 2007). Co-stimulation with GpC-ODN and plasmid resulted in an approximately two-fold lower induction than that of CpG-ODN and plasmid. That a more potent induction of immune responses was observed with CpG- rather than CpG-ODNs reflects the cooperative effect of plasmid-mediated activation by CpG-ODNs.

4.4 Establishment of a TLR9 Knockout Cell Line

Establishment of a TLR9$^{-/-}$ cell line will allow us to express various forms of TLR9, either wild-type and mutant, in order to determine cellular responses to various ligands. Fibroblast cells normally express very low levels of TLR9, which explains their unresponsiveness to TLR9 ligands (Hornung, Rothenfusser et al. 2002; Kyburz, Rethage et al. 2003). Murine embryonic fibroblasts respond normally to TLR1, TLR2, TLR4 and TLR6 ligands, suggesting that the TLR signaling infrastructure, absent TLR9, is present in these cells (Satta, Dunoyer-Geindre et al. 2007).

The TLR9$^{-/-}$ fibroblasts were isolated from tissue isolated from the abdominal wall tissue of TLR9$^{-/-}$ mice. The initial cell population included myocytes (muscle cells) as well as fibroblasts. Cells were maintained and passaged in 10% FBS for one week prior to being supplemented with 20% FBS-containing media. Within four weeks of initial culture, cells developed fibroblast-like foci and approximately two weeks later, cells developed contact inhibition and were increasing in density. Seven weeks after initial culture, fibroblasts were the dominant cell type.

Using these knockout cells should provide us with a TLR9-null background, which will allow us to measure the responses of TLR9 to ligand stimulation with the appropriate negative control.

The cells had no detectable TLR9 protein expression, as verified via traditional Western blot analysis against proteins purified from cell extracts using TRIzol methods. There was no reaction between the α-human TLR9 monoclonal antibody and the proteins purified from the $TLR9^{-/-}$ cells. Sequence alignment data (not shown) indicates that the epitope to which the antibody binds is 100% homologous between human and murine TLR9.

$TLR9^{-/-}$ mice generated by Hemmi and his group were genotyped and verified to be TLR9-deficient (Hemmi, Takeuchi et al. 2000; Babiuk, Mookherjee et al. 2004). The mutated TLR9 allele was PCR amplified using specific primers, and it was confirmed that mice did not express TLR9 (Babiuk, Mookherjee et al. 2004).

4.4.1 Transfections

Transfection efficiency was assessed using a fibroblast transfection reagent and a GFP-expressing plasmid, and was optimized by testing different parameters. The recommended amount of transfection reagent is 3.5 μL and the recommended amount of DNA for transfection is 500 ng. Using these parameters, some transfection was observed, though it was well under the stated 90% that could be achieved under recommended parameters (**Table 4.2**). Therefore, the amount of reagent and the amount of DNA were varied in an attempt to optimize transfection. Transfection efficiency increased from ~2% to approximately 30-40% as parameters were optimized (**Figure 4.8**). Below the recommended volume of transfection reagent, transfection efficiency was very low, with almost no transfection seen at all with increasing concentrations of DNA. While 500 ng DNA was the recommended amount of DNA to be used, 600 ng

Table 4.2. Parameters tested for transfection of TLR9-/- fibroblast cells.
Results indicated that 600-800 ng of DNA transfected with 3.5-4.5 µL of transfection reagent was the most efficient (++). Below 3.0 µL of transfection reagent, transfection was negligible (-/+). n/a indicates that transfections were not carried out under those conditions.

Concentration of DNA (ng)	**Volume of transfection reagent (µL)**						
	2.0 µL	**2.5 µL**	**3.0 µL**	**3.5 µL**	**4.0 µL**	**4.5 µL**	**5.0 µL**
500 ng	-/+	-/+	+	+	+	+	+
600 ng	-/+	+	+	++	++	++	+
800 ng	n/a	n/a	+	++	++	++	+
1000 ng	n/a	n/a	n/a	+	+	+	+

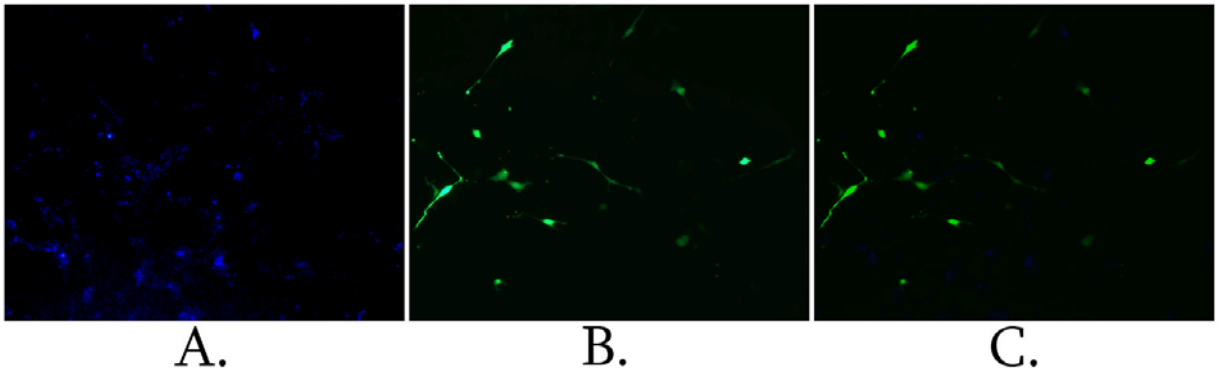

Figure 4.8. Transfection of TLR9$^{-/-}$ fibroblast cells. A) DAPI stained nuclei. Cells were rinsed 2X with PBS and incubated with 0.5mL 300nM DAPI/well for 10minutes at room temperature. Cells were then rinsed 2X with PBS and visualized under fluorescence microscope. **B)** GFP-expressing cells. Cells were visualized using a FITC filter on the fluorescence microscope. **C)** Merging DAPI and GFP images.

and 800 ng showed more efficient transfection at 3.5-4.5 μL transfection reagent. Increasing the amount of transfection reagent above 4.5 μL did not improve transfection. Though transfection efficiency has increased from ~2% to approximately 30-40%, it is still rather low. Currently, we do not have the protocols in place to select for transfected cells. Should transfection efficiency fail to increase to a level suitable to our needs, selection procedures will be the next avenue to pursue.

5.0 DISCUSSION AND CONCLUSIONS

TLR9 has attracted considerable attention for its role in the initiation of innate immune responses and for the ability of synthetic nucleic acids to modulate TLR9 signaling. TLR9 agonists are under active investigation for a variety of clinical applications. Given the emerging promise of this class of molecules, coupled with their potential to induce damaging consequences such as autoimmune disorders, it is timely and appropriate to characterize the structural parameters that define the TLR9-ligand interaction in order to facilitate the design of more efficient and specific agonists.

The immunostimulatory action of bacterial DNA can be effectively mimicked with synthetic, single-stranded ODNs. As therapeutic TLR9 agonists these ODNs have considerable advantage over bacterial DNA as a result of their defined sequences and structures, their low costs of synthesis, and chemical stability, however they do require relatively high doses to induce innate immune responses and co-formulation is often necessary.

CpG-ODNs are structurally complex molecules with numerous regions, including the nitrogenous bases, sugar groups and phosphodiester linkages which have the potential to influence their ability to serve as TLR9. Modifications to the 2' position of the sugar group (2'-O-methyl, 2'-O-methoxyethyl, for example) have been shown to decrease the effectiveness of ODNs, while modifications to the phosphodiester linkages

(addition of hydroxy, alkyl, or methyl groups) have also been shown to decrease the activity of ODNs (Vollmer, Weeratna et al. 2004; Kalota, Dondeti et al. 2006). To elucidate the structure/immunological function relationship of TLR9 agonists, investigations have been conducted looking at how alterations of different regions of these molecules influence their ability to activate TLR9-dependent responses.

The low cost, ease of production and stability of these DNA ligands make TLR9 an attractive target amongst the TLRs for immunotherapeutic intervention. The ability of CpG treatment to modulate immune responses has given rise to a number of therapeutic applications including: 1) priming the innate immune system to mediate host protection, 2) as adjuvants to promote induction of antigen-specific immune responses, 3) as anti-allergens through establishment of T_H1 responses, 4) in the treatment of a variety of malignancies and 5) for improved vaccination efficiency of individuals with poor immune responses (Zimmermann, Egeter et al. 1998; Krieg, Yi et al. 1999; Dittmer and Olbrich 2003; Klinman 2004; Lazarczyk, Grzela et al. 2005). As immunoprotective agents, CpG ODNs trigger plasmacytoid dendritic cells to secrete type I IFNs, which are known to limit the growth of bacteria and viruses by activating macrophages. This is the beginning of a complex cascade which includes activation of natural killer (NK) cells. Innate immune responses elicited by CpG DNA improve host resistance to infection.

Currently, however, there is no structural ligand-binding information available for TLR9. Crystallographic studies have been performed for the ectodomain of human TLR3, which has high sequence homology to TLR9 and also binds unmethylated nucleic acids within the acidic endosomal compartments. However, rather than

resolving the mechanisms of nucleic acid recognition, these studies have proposed two conflicting models of ligand binding; Choe and coworkers propose the convex side of a dimerized structure to be the ligand-binding region (Choe, Kelker et al. 2005), while Bell and coworkers propose that a binding groove for double-stranded RNA lies within the inner cavity of a TLR3 monomer (Bell, Botos et al. 2005). To date, there is no definite model of ligand binding for TLR3, or for TLR9.

Thus, little consensus has been reached with regard to: 1) the efficiency of TLR9 in binding dsDNA, 2) the requirement of CpG motifs for mediating ligand binding events, 3) the ability of TLR9 to recognize higher order motifs (beyond the CpG motif) in a species-specific fashion, 4) the influence of the phosphorothioate modification on receptor-ligand binding and 5) whether ligand binding is influenced by higher order mechanisms. Our goal was to use recombinant TLR9 protein to address these issues.

5.1 Ligand Binding Properties of Recombinant Toll-like Receptor 9

Ligands which activate TLR9 have been identified, and there is a basic understanding of the TLR9 signaling cascade. Upon binding of the ligand to TLR9, the adaptor molecule MyD88 is recruited, via the TIR domain, leading to subsequent phosphorylation of IRAKs and IKKs. This ultimately leads to the production of inflammatory cytokines by NF-κB and IFNα/β (and IFN-inducible genes) by IRF7. Characterization of the ligand-binding properties of TLR9 has been limited by the difficulty of purifying the receptor in sufficient quantity for biochemical analyses, and the absence of convenient screening assays. Although cell stimulation experiments

provide valuable information regarding the *in vivo* interactions between biomolecules based on cellular responses, a danger of this type of approach is that complex physiological signals and responses are reduced to a single measured output which may overlook potential points of regulation within the system, such as phosphorylation events, interactions with adaptor molecules and other secondary signaling molecules, as well as any response generated that is different than the output being measured, making it difficult to reach conclusions with respect to ligand binding.

In order overcome these problems, we used novel methods for the purification of the soluble, ligand-binding domain of human TLR9 and a convenient, high-throughput assay that permits comparative analysis of nucleic acid binding. An advantage of the MDBK cell expression system is that the recombinant protein is secreted into the media following expression, thus eliminating the requirement for a cell lysis step. This decreases the potential for co-purification of contaminating proteins which may influence characterization of TLR9(LBD). Gel shift assay systems have a distinct advantage over surface plasmon resonance (SPR), the technique utilized by other *in vitro* investigations of TLR9 ligand binding, in that they do not require immobilization of either the receptor or the ligand.

Using the present system we demonstrated stable nucleoprotein complex formation between the recombinant TLR9(LBD) and plasmid DNA molecules. While it has been demonstrated that innate immune responses can be initiated by plasmid DNA (Krieg, Yi et al. 1995) and time course experiments verify that plasmids remain intact within the endosomes for a sufficient duration to serve as TLR9 ligands (Bennett, Gabor et al. 1985), previous investigations have reached opposing conclusions with respect to

the ability of TLR9 to bind plasmids. While a system established by Cornélie and his group demonstrated effective binding of plasmids (Cornélie, Hoebke et al. 2004), Rutz reported a weak interaction between TLR9 and double-stranded ligands (Rutz, Metzger et al. 2004). Our observation that the receptor undergoes preferential association with supercoiled species may account for the differing conclusions, and suggests that TLR9 does bind dsDNA. It was confirmed that the receptor was indeed binding supercoiled DNA by extraction of DNA from the nucleoprotein complex. Isolated DNA was run through an agarose gel and migrated as a supercoiled species (data not shown). In the investigation by Rutz, double-stranded ODNs, rather than plasmids, were employed as ligands. The inability of double-stranded ODNs to form supercoiled species may account for their reduced efficiency as ligands. In addition, SPR analysis requires immobilization of the receptor or ligand. This immobilization may influence the receptor:ligand interaction and potential receptor:receptor interactions by limiting the range of conformations that the bound molecule could adopt, meaning that receptor dimerization could not occur.

5.1.1 Sequence and Structural Specificity

From investigations of immune cell responses the CpG motif has emerged as the important feature for TLR9-mediated cellular activation. Investigations by Cornélie and Rutz have demonstrated a preference for CpG- rather than GpC-ODNs but reached no conclusions regarding the ability of the receptor to bind double-stranded nucleic acids (Cornélie, Hoebke et al. 2004; Rutz, Metzger et al. 2004). It is generally assumed that this specificity for the motif is determined at the level of ligand binding by TLR9.

While CpG-specific activation of the innate immune responses has been assumed to reflect sequence-specific binding by TLR9, recent binding assays have reached contradictory conclusions with respect to the ability of the receptor to associate with nucleic acids in a sequence-specific manner (Cornélie, Hoebke et al. 2004; Latz, Schoenemeyer et al. 2004; Rutz, Metzger et al. 2004; Shirota, Gursel et al. 2004). Our investigations may offer an explanation for this apparent discrepancy by demonstrating the ability of TLR9 to form both stable and highly sequence-specific nucleoprotein complexes, but also to be sensitized by nucleic acids in a sequence-independent fashion.

Using *in vitro* binding assays, the ability for TLR9(LBD) to discriminate the CpG motif, as well as higher order sequences of six base pairs in length has been demonstrated. The six base pair CpG motif preferentially bound by the recombinant human TLR9(LBD) in the presence of plasmid corresponded with the sequence that elicits the greatest *in vivo* responses in humans, GTCGTT. In contrast, the murine motif, GACGTT, did not form nucleoprotein complexes with the recombinant receptor in the presence of plasmid (Kindrachuk, Potter et al. 2007). As such, formation of stable complexes is highly sequence-dependent and this may dictate the specificity of activation of the innate immune system.

5.1.2 Interaction versus Activation

The sequence-specificity of the formation of stable nucleoprotein complexes suggests that the interaction between TLR9 and nucleic acids is sequence-dependent. However, that TLR9 has the ability to be functionally influenced by inhibitory nucleic acid sequences implies that the receptor has the ability to bind both stimulating and non-

stimulating sequences, possibly highlighting the functional distinction between ligand binding and receptor signaling. This is of particular consequence as it limits the potential to discover novel agonists when using a screening assay which only measures the ability an of agonist to interact with its receptor.

In contrast to the sequence specificity of nucleoprotein complex formation our results also demonstrated the ability for both single and double-stranded nucleic acids to exert a cooperative effect on TLR9 ligand-binding in a sequence-independent fashion. In the presence of ODN, TLR9 is more efficient in binding plasmid and vice versa. This effect is hypothesized to result from the requirement of TLR9 to form dimers in order to sample potential nucleic acid ligands for the presence activating motifs. While non-activating nucleic acids are rapidly released, the dimer formed persists momentarily and, during this time, is activated to bind other prospective ligands. Others have noted that TLR9 displays low affinity binding to ODNs which is enhanced by the presence of CpG motifs, or increased ODN concentration, to a higher avidity form of the receptor (Yasuda, Rutz et al. 2006). This observation is consistent with the model of cooperativity that we propose.

5.1.3 Model of Cooperativity

Using the model of cooperativity, a number of key issues with respect to the sequence and structural parameters that influence the ability of nucleic acids to function as TLR9 ligands can be resolved. Sequences which are unable to mediate direct activation of TLR9 appear to influence the threshold of TLR9 activation by exerting a

positive cooperative effect on ligand binding *in vivo* and *in vitro*. This is in contrast to the model of negative cooperativity shown with *Drosophila* Toll.

It is hypothesized that the nucleoprotein complexes formed during the agarose shift assays correspond to monomeric and dimeric forms of the receptor (**Figure 4.5**). Dimerization is a common theme in the Toll and TLR family of receptors (Gangloff and Gay 2004; Weber, Moncrieffe et al. 2005). These results may highlight the difference between activation and interaction. Cell stimulation experiments demonstrate that CpG- rather than GpC-containing ODNs preferentially activate the innate immune system, which would suggest that nucleic acids bind TLR9 in a sequence-dependent manner. Conversely, immunoprecipitation experiments have shown that both CpG- and GpC-containing ODNs interact with TLR9, suggesting that nucleic acid binding to TLR9 is sequence-independent (Krieg, Yi et al. 1999; Latz, Schoenemeyer et al. 2004; Rutz, Metzger et al. 2004). Our observations that ODNs and plasmids, independent of their sequence, functionally influence TLR9 ligand binding are consistent with the conclusion that TLR9 has the ability to interact with, and be modulated by, nucleic acids in a sequence-independent fashion.

Activation of Toll is proposed to begin with the existence of an inactive ligand-free dimer and proceed through a two-step mechanism in which the formation of the active dimer is preceded by the formation of a monomeric ligand complex. Spätzle, the ligand of Toll, also functions as a homotropic allosteric inhibitor to exert negative allosteric effects on formation of the active dimer. Spätzle, upon binding to the active site of the first monomeric subunit of Toll, induces structural alterations which are translated to the second subunit via non-covalent associations. These alterations reduce

the affinity of the second subunit for Spätzle, causing the second step to Toll activation to occur with an approximately three-fold lower affinity than the binding of the Spätzle by the first subunit (Gangloff, Weber et al. 2003; Gay, Gangloff et al. 2006). This effect was reported as negative cooperativity and is proposed to increase the range of substrate concentrations to which Toll is responsive. This mechanism has been verified *in vitro* at the level of ligand binding, as well as through kinetic characterization. The authors hypothesized a similar mechanism may influence the activation of the Toll-like receptors (Gay, Gangloff et al. 2006).

In contrast to the negative cooperativity reported for Toll, we have observed the ability for both single and double-stranded nucleic acids to exert a positive cooperative effect on TLR9 ligand binding; in the presence of ODNs, TLR9 is better able to form nucleoprotein complexes with plasmid molecules and conversely TLR9 is also better able to bind ODNs in the presence of plasmid. This mutual allostery occurs in a sequence-independent fashion as CpG- and GpC-ODNs are equally effective in promoting plasmid binding by TLR9. As described, this is hypothesized to result from the requirement of TLR9 to form dimers to sample potential nucleic acid ligands in order to discriminate activating from non-activating nucleic acids. While non-activating nucleic acids are rapidly released by TLR9, the dimer persists and is activated with a higher affinity for binding of other nucleic acid ligands (**Figure 5.1**).

The schematic presented illustrates the proposed equilibrium between the TLR9 monomer, occupied dimer and ligand-free dimer. This model requires TLR9 to associate with nucleic acids in a sequence-dependent fashion, at least momentarily. The associations with non-activating ODNs are sufficient to cause dimer formation and shift

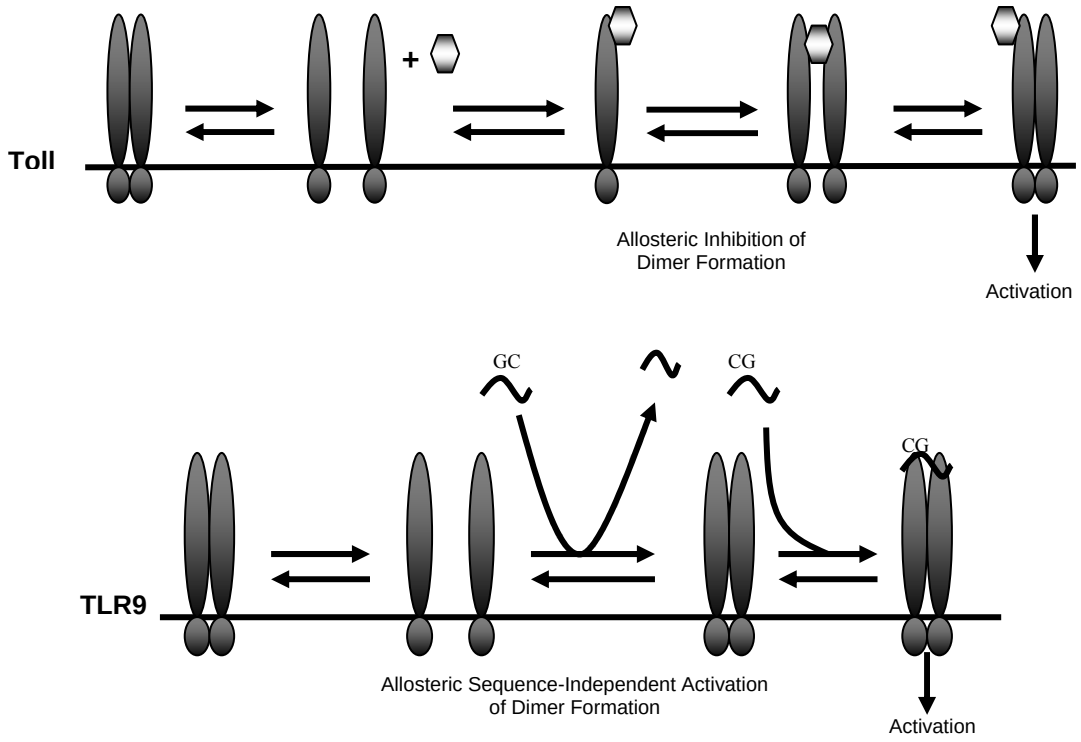

Figure 5.1. Models of activation of Toll and Toll-like Receptor 9. Activation of Toll is mediated through a two-step reaction. The first binding event, to the formation of a ligand-bound monomer, occurs with high affinity. The second stage of Toll activation, binding of the Toll-ligand monomer by a ligand-free monomer, occurs with lower affinity, representing negative cooperativity of activation. Activation of TLR9 is proposed to follow a similar two-step reaction with the distinction being that the second step to dimer formation occurs with positive cooperativity.

the monomer-dimer equilibrium of TLR9 to the active form of the receptor. Notable for TLR9 we did not observe the presence of labeled ODNs in plasmid:TLR9 nucleoprotein complexes which indicates that the ability to promote this complex formation does not involve the simultaneous binding of each type of nucleic acid. For both Toll and TLR9, the allosteric modulators are also ligands in the sense that they are bound at the active site and as such represent homotropic modulators.

Following the release of non-activating sequences, the ligand-free TLR9 dimer persists briefly. Though this dimer is inactive, it has been sensitized and has an increase affinity for nucleic acids. The binding of activating sequences by the ligand-free dimer would be hypothesized to induce structural alterations within the TIR domains of the TLR9 dimer leading to receptor activation. In contrast to the Toll model, the conformational changes induced within TLR9 following the sequence-independent interaction with nucleic acids increase the affinity of the receptor for the stable binding of activating or non-activating sequences. This results in the second step of TLR9 activation proceeding with a much higher affinity than the first step. The equilibrium between TLR9 monomers, ligand-bound dimers, and ligand-free TLR9 dimers is illustrated in **Figure 5.1**. Supportive of this model of higher order functional influence of nucleic acids on TLR9 activation, the sequence-independent association of DNA with TLR9 has become been independently confirmed (Latz, Schoenemeyer et al. 2004; Yasuda, Rutz et al. 2006).

Collectively these results demonstrate that nucleic acids influence TLR9 functioning in both sequence-dependent and sequence-independent fashions. While

the binding of ligands for activation of receptor signaling is highly sequence-specific, non-activating nucleic acids are able to modulate TLR9 ligand binding by altering ligand binding affinity. That ODNs are able to modulate TLR9 function through influencing sensitivity helps to establish the ability of these molecules to interact with, but not directly activate, TLR9. Taken together with the observations of Toll, this suggests that both of these receptor families activate through a two-step process with the initial binding event determining specificity while the second binding event dictates sensitivity in a positive or negative fashion.

The physiological significance of this cooperative effect may be in determining the set point for activation of TLR9. That increased quantities of nucleic acid are able to sensitize the system in a sequence-independent fashion suggests that release of nucleic acids following bacterial uptake within the phagosome primes the system for innate immune responsiveness if activating CpG motifs are present. This dual requirement for activation may function as a safe guard to prevent the induction of inappropriate innate immune responses and for discrimination of self from non-self.

The proposed model of cooperative sensitization of TLR9 is supported by cell stimulation experiments where the magnitude of proliferation responses to plasmid stimulation in the presence of either stimulatory or non-stimulatory natural ODNs was greater than the sums of activation that were elicited by either of these classes of molecules in isolation (Kindrachuk, Potter et al. 2007). A recent report by Latz *et al* also supports these findings. Using biochemical and fluorescence resonance energy transfer, fluorescence lifetime imaging microscopy and circular dichroism spectroscopy, they found that their recombinant TLR9 bound stimulatory and inhibitory DNA but only

stimulatory DNA led to conformational changes in the ectodomain of the receptor (Latz, Verma et al. 2007). Activation of TLR9 is said to be regulated by conformational changes in the ectodomain induced by CpG-containing DNA. This is consistent with our findings that TLR9 binds DNA in a sequence-independent manner but is activated in a sequence-dependent manner.

5.2 CpG ODNs

The observation that TLR9 can be functionally influenced by non-CpG elements expands the range of nucleic acids that can be employed to modulate innate immune responses through TLR9. The design of novel TLR9 immunotherapeutics may benefit from consideration of TLR9 modulators, rather than direct activators. These molecules would be selected on the basis of the ability to prime the TLR9 system for CpG-specific activation with the potential for co-formulation of activating and modulating molecules.

5.3 Future Applications

Attention has been brought to the therapeutic potential of TLR9 activation through various mechanisms including, but not limited to, innate immune activation with T_H1-like cellular and cytokine/chemokine responses, as well as enhancing antigen-specific humoral and cellular adaptive immune responses. In spite of the considerable therapeutic potential of CpG-ODNs to activate TLR9, reasonable concerns have been raised with regards to their safety, particularly local inflammation, autoimmunity and excessive immune stimulation.

Current data would suggest that the receptor binds several motifs besides the natural CpG motif, and that these alternative recognition patterns produce different cytokine-secretion profiles. Investigations of molecular recognition of modified CpG DNAs by different cells from a wide range of species would further define the process, and lead to the design of less toxic immunomodulators for therapeutic applications.

Using our system of soluble TLR9(LBD) expression, we have developed a model which resolves a number of key issues with respect to the sequence and structural parameters that influence the ability of nucleic acids to function as TLR9 ligands, and permits comparative analysis of nucleic acid binding by the receptor. The use of the heat shock system of expression of the recombinant receptor and the development of a convenient assay to screen for potential TLR9 ligands could advance the process of developing TLR9 agonists. We have in place a system to determine not only the ability of ligands to bind the receptor, but also the ability of ligands to form stable complexes with the receptor for activation. We have determined that the recombinant TLR9(LBD) binds ligands in a sequence-independent manner and, in contrast, presume that receptor activation is highly dependent on sequence. Ligand binding by non-activating nucleic acids appears to be an important step in sensitization of the receptor to activating nucleic acids, as it has been demonstrated that, in the presence of plasmid, CpG-containing ODNs cause an increase in cell proliferation compared to GpC-containing ODNs (Kindrachuk, Potter et al. 2007). These results are consistent with a TLR9-type response, though TLR9 is not the only TLR expressed in PBMCs (Armstrong, Medford et al. 2004; Werling, Hope et al. 2004). Using the $TLR9^{-/-}$ fibroblast cell line, we could investigate the effect of activating and non-activating nucleic acids on a TLR9-null

background as a control. To determine the role played by TLR9, the cells could be transfected with a TLR9 construct and assessed as such using activating and non-activating nucleic acids.

Though there is increasing evidence for cross-talk among various cell signaling pathways, such as B cell receptors and cytokine receptors (Hill 1998; Guo, Blair et al. 2007), to date there is no evidence to suggest that TLR9 interacts directly with other receptors. Our system would allow determination of not only receptor-receptor interaction, but also stable complex formation between receptors. Pattern recognition receptors, including TLRs, are usually shared by cells of the innate immune system, including polymorphonuclear phagocytes, monocytes/macrophages, DCs and natural killer cells, though TLR9 is said to be confined to B cells and plasmacytoid dendritic cells in resting human immune cells (Krieg 2006). All of these are cells with specialized function which cooperate to mount effective immune responses. In theory, receptors could cross-talk with one another to increase and diversify recognition of microbial infection, as is the case with other members of the TLR family, including TLRs 1, 2 and 6.

Use of this system of expression is not limited to the TLR9(LBD). The expression system can be modified to express any, or all, of the TLRs in order to elucidate their ligand binding properties as we have done with TLR9. The ligand binding properties of the other nucleic acid binding TLRs (3, 7 and 8) could be investigated by a similar means as the TLR9(LBD), while other protein-binding receptors could be expressed using this system and investigated by other means. Investigation into the ligand binding properties of other TLRs is of particular importance

due to their roles in the induction of the innate immune system in response to a wide variety of pathogens. Though there is an incredible amount of redundancy built in to the TLR signaling cascades, there is also a tremendous amount of specificity. As such, it would be valuable to look beyond TLR9 as a potential for therapeutic intervention.

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